Limnanthes douglasii lysophosphatidic acid acyltransferases: immunological quantification, acyl selectivity and functional replacement of the *Escherichia coli* *plsC* gene

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Antibodies were raised against the two membrane-bound lysophosphatidic acid acyltransferase (LPAAT) enzymes from *Limnanthes douglasii* (meadowfoam), LAT1 and LAT2, using the predicted soluble portion of each protein as recombinant protein antigens. The antibodies can distinguish between the two acyltransferase proteins and demonstrate that both migrate in an anomalous fashion on SDS/PAGE gels. The antibodies were used to determine that LAT1 is present in both leaf and developing seeds, whereas LAT2 is only detectable in developing seeds later than 22 daf (days after flowering). Both proteins were found exclusively in microsomal fractions and their amount was determined using the recombinant antigens as quantification standards. LAT1 is present at a level of 27 pg/μg of membrane protein in leaf tissue and ≤ 12.5 pg/μg of membrane protein in developing embryos. The amount of LAT2 reaches a peak at

305 pg/μg of membrane protein 25 daf and is not expressed 20 daf or before. This is the first study to quantify these membrane-bound proteins in a plant tissue. The maximal level of LAT2 protein coincides with the maximal level of erucic acid synthesis in the seeds. Both full-length proteins were expressed in the *Escherichia coli* LPAAT mutant JC201, and membranes from these strains were used to investigate the substrate selectivity of these two enzymes, demonstrating that they are different. Finally, we report that LAT2 and a maize LPAAT enzyme (MAT1) can functionally replace the *E. coli* *plsC* gene after its deletion in the chromosome, whereas LAT1 and a coconut LPAAT (Coco1) cannot. This is probably due to differences in substrate utilization.

Key words: acyl-CoA, antibody, complementation, membrane.

INTRODUCTION

In plants, the *de novo* biosynthesis of both phospholipids and triacylglycerols (TAGs) from glycerol 3-phosphate involves two sequential acylation reactions catalysed by glycerol 3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT) respectively to produce phosphatidic acid (PA). During TAG biosynthesis, after de-phosphorylation of the PA, a third acyl-CoA-dependent acyltransferase, diacylglycerol acyltransferase, acylates diacylglycerol to form TAG. Both soluble and membrane-bound forms of GPAT exist in plants. The soluble form of GPAT, located in the plastid, uses acyl-carrier protein substrates and has been purified, cloned and overexpressed [1]. Although our knowledge of the soluble enzyme is quite advanced, including its structural determination [2], to date no membrane-bound form of GPAT has been isolated to homogeneity from any plant source. Partial purification of membrane-bound GPAT from plants has been described previously [3,4].

Membrane-bound enzymes, including GPATs and LPAATs, are difficult to purify due to their hydrophobic nature, tendency to aggregate and loss of activity during purification. To overcome this, other genetic-based techniques have been applied to identify and clone them. The first cDNA for a plant LPAAT, MAT1, was isolated from maize by complementation of the temperature-sensitive *Escherichia coli* LPAAT mutant JC201 [5]. cDNAs encoding LPAATs from a number of diverse species have been shown to complement JC201, including those from human, mouse and plant sources [6–8]. *Limnanthes douglasii* (meadow-

foam) has two different LPAAT genes, *LAT1* and *LAT2*. The latter was cloned by complementation of JC201 and the former by hybridization to the MAT1 cDNA [8]. Attempts to clone other potentially important plant LPAATs such as the one from coconut endosperm [9] did not succeed using complementation, indicating the potential limitations of this technique that are probably based on biochemical compatibility.

The main driving force for cloning plant LPAAT genes was the observed difference in LPAAT substrate selectivity in different species [10,11]. Oil seed rape does not contain an LPAAT that can utilize erucic acid (22:1 Δ13), placing a limitation on the TAG molecular species which can be synthesized, as erucic acid cannot be incorporated into position 2 of TAG. The LPAAT in the seed of *Limnanthes* spp. however can use erucic acid and as part of programmes to introduce LPAATs with different substrate selectivity into rape (enabling them to make potentially new oils) the cloning of LPAAT from *L. douglasii* was instigated. Two different LPAAT clones were isolated from this species, one encoding a protein with a predicted molecular mass of 42.7 kDa (LAT1), and the second encoding a protein with a predicted molecular mass of 31.6 kDa (LAT2) [8,12]. Comparison of the sequences of these proteins showed that they had very little similarity across the whole protein (< 15%), and the only common features were at the putative active-site domains, which were conserved in all acyltransferases [13]. In these domains, there was 60% similarity between LAT1 and LAT2 over ten amino acids. Northern-blot analysis showed that LAT1 is expressed in leaf and developing embryos, and LAT2 is expressed in embryos but not in leaves, consistent with a role for

Abbreviations used: daf, days after flowering; DTT, dithiothreitol; GPAT, glycerol 3-phosphate acyltransferase; IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame; LPAAT, lysophosphatidic acid acyltransferase; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; PA, phosphatidic acid; Pefabloc, 4-(2-aminoethyl)benzenesulphonylfluoride hydrochloride; TAG, triacylglycerol; TBS, Tris-buffered saline.

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LAT2 in TAG assembly. Such analyses cannot give information regarding the amount of LAT1 or LAT2 protein in different tissues, as this is dependent on mRNA turnover, transcription rate and protein stability. Few studies have attempted to determine the level of membrane-bound proteins, such as acyltransferases, in plant tissues. There may be physiological limits to the level of incorporation of such proteins into membranes. Determination of the levels of these enzymes may therefore be important in designing new genetically modified crops. To determine the level at which acyltransferases are present in the membranes of plant material, a direct measurement is required.

Both LAT1 and LAT2 are predicted to be integral membrane proteins. To determine their expression levels within membranes and to facilitate further studies on their regulation and sub-cellular localization, specific antibodies are required. The difficulties inherent in expressing membrane proteins in *E. coli* are well known [14]. Frequently, membrane proteins fail to express to a significant level, and researchers have resorted to using anti-peptide antibodies, which tend to have low titre and can cross-react with other proteins [15]. An alternative strategy is to express the protein without its hydrophobic domains, and we report here the use of this technique to raise antibodies against LAT1 and LAT2.

LPAATs function during the synthesis of membranes and storage oil, and they have also been shown to be important in Golgi vesicle membrane fission [16]. *L. douglasii* is the only plant species for which separate LPAAT genes, presumably associated with membrane and seed storage oil production were isolated. This makes *L. douglasii* an interesting model species for the study of the control and compartmentalization of lipid production within the endoplasmic reticulum. The role of LAT2 in TAG biosynthesis was shown by transformation of LAT2 in oil seed rape. It resulted in the incorporation of 22:1 at the *sn*-2 position of TAG [17,18]. It is apparent that LAT2 can utilize 22:1 as a substrate but little is known about which acyl groups LAT1 will utilize.

Isolation of plant cDNAs by complementation of *E. coli* mutants, as in the cloning of LAT2 [8], is a powerful technique but it does not show that the isolated gene can completely and functionally replace its *E. coli* counterpart, proving its biological activity. There are several cases where complementation of a mutant can be achieved by a gene that is functionally different. For example, the MCAT mutant of *E. coli* is complemented by a putative GTP-binding protein [19]. Formative proof of function is obtained by complete deletion of the host gene and replacement with a putative homologue [20]. The success of this procedure may be limited due to specific differences in substrate preference, correct protein folding and integration into the *E. coli* hosts metabolic machinery. We have therefore investigated whether the *E. coli* LPAAT gene, *plsC*, can be deleted and functionally replaced by plant homologues.

In the present study, we had the following objectives: (1) to see if both LAT1 and LAT2 plant LPAATs can completely replace the *plsC* gene using an *E. coli* deletion mutant, (2) to raise antibodies that can discriminate between LAT1 and LAT2, and use them to measure protein levels in both leaf and developing seed material, (3) to investigate the substrate selectivity of LAT1 and LAT2 with respect to the acyl groups and (4) to correlate these findings with the lipid profiles of developing *L. douglasii* embryos.

EXPERIMENTAL

Plasmids used for complementation experiments

Plasmids pLAT1, pLAT2 and pMAT1 were described previously [5,8]. The *E. coli* LPAAT gene *plsC* was present on the plasmid

pPLSC [21], and this plasmid complemented the temperature-sensitive *E. coli* LPAAT mutant JC201 [22], as do pMAT1 and pLAT2. A *Neisseria meningitidis* LPAAT gene [23] was amplified by PCR and cloned into the *EcoRI* site of the temperature-sensitive plasmid pMT101 [24]. The primers used were (N-terminal) 5'-CGGAATTCTCTAGAGGGGTATTAATAATGTCTTCAAATAAGCTTCAT-3' and (C-terminal) 5'-CGGAATTCTCAGACAGCGATATCCGCATC-3'. The amplified fragment contained an *E. coli* lipoprotein translation initiation signal to ensure efficient translation of the LPAAT; the resulting plasmid, pMT149, conferred tetracycline resistance. The coconut LPAAT sequence described previously [9] was obtained by reverse transcription PCR using RNA from developing coconut endosperm and cloned as a *NcoI/BamHI* fragment into the expression plasmid pQE-60 (Qiagen, Crawley, Sussex, U.K.) to give pQE-60Coco. Sequences encoding LAT1 and LAT2 from *L. douglasii* were similarly cloned into pQE-60, after PCR using pLAT1 and pLAT2, to give pQE-60LAT1 and pQE-60LAT2 respectively.

Preparation of truncated antigens and immunization

For expression of truncated LAT1 protein lacking the hydrophobic N- and C-termini, a 25.3 kDa portion of the cDNA (encoding amino acids 81–304) was cloned as an *NcoI-BglII* fragment into the expression vector pET-11d via PCR. The primers used were (N-terminal) 5'-CATGCCATGGGTAAAGAACATGCAC-3' and (C-terminal) 5'-GAAGATCTCACTTCATTGGCCGACCAATGTC-3'. For expression of truncated LAT2 protein lacking the hydrophobic N-terminal, a 21 kDa portion of the gene (amino acids 97–281) was cloned as an *NcoI-BamHI* fragment into the expression vector pET-11d via PCR. The primers used were (N-terminal) 5'-CATGCCATGGAGCATACAAAGAAGAGGGC-3' and (C-terminal) 5'-TCGGATCCTCACTTTGAGCGATTGTGCTAC-3'. The constructs, pETLAT1 and pETLAT2 respectively, were transformed into *E. coli* strain BL21(DE3) (Novagen) and truncated proteins expressed in bacteria grown in 2YT medium using 0.5 mM isopropyl β -D-thiogalactoside (IPTG) induction for 3 h at 37 °C. The induced cells were lysed in 50 mM Tris/HCl, pH 8.0, using a cell disrupter (Constant Systems Ltd., Warwick, U.K.) and the cellular debris, including the majority of the expressed proteins which were concentrated within inclusion bodies, collected by centrifugation at 5000 *g* for 10 min at 4 °C. After washing the pellet in 2 M NaCl, the proteins were solubilized in 1 % SDS and purified in a Bio-Rad Prep Cell according to the manufacturer's instructions. Excess SDS was removed and the proteins were concentrated by passage through an Amicon Centrprep concentrator (10 kDa molecular mass cut-off). Antisera were raised (Serotec, Kidlington, Oxford, U.K.) in rabbits using 100 μ g protein in Freund's complete adjuvant as first inoculum and 5 $\times$ 35 μ g of protein in Freund's incomplete adjuvant for subsequent fortnightly boosts.

Affinity purification of antisera

Pellets containing inclusion bodies of LAT1 and LAT2 from 2 litre cultures were resuspended in 25 ml of 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mg/ml deoxycholate, 0.2 mg/ml lysozyme, 0.2 mg/ml DNase I, 0.1 mM Pefabloc [4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride; Boehringer Mannheim]. After 30 min incubation at room temperature (20 °C) the inclusion bodies were recovered by centrifugation at 20000 *g* for 20 min at 4 °C and then resuspended in 40 ml of 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 2 M NaCl and 0.1 mM

Pefabloc. After a further 30 min incubation on ice, the inclusion bodies were collected by centrifugation at 20000 *g* for 20 min at 4 °C. The washed inclusion bodies were soaked overnight at room temperature in 5 ml of solubilization buffer [50 mM Tris/HCl, pH 8.0, 6 M guanidine/HCl, 1 mM EDTA, 5 mM dithiothreitol (DTT)]. Purification was achieved by size-exclusion chromatography in a 30 cm × 1 cm Superose column run at 0.5 ml/min in solubilization buffer. Purified antigens were quantified with Bio-Rad protein assay reagent using ovalbumin as standard. Affigel-10 (Bio-Rad Laboratories, Hemel Hempstead, U.K.) immuno-affinity columns (2 ml) for each antigen were prepared according to the manufacturer's instructions using 5 mg of antigen in 1 M guanidine/HCl, pH 7.5. The first stage in specific antibody purification was to purify the IgG fraction from the serum on a DEAE Affigel Blue column (Bio-Rad Laboratories) according to the manufacturer's instructions. Serum samples (5 ml) were used in each case on a column with 60 ml bed volume. Eluted IgG fractions were concentrated to 5 ml using an Amicon concentrator containing a PM30 membrane. This entire concentrated IgG fraction was diluted with 2 vol. of 1 × PBS containing 0.02 % sodium azide and loaded on to the relevant Affigel-10 immuno-affinity column under gravity. The eluate was recycled through the column a further six times before being discarded. Columns were washed with 100 ml 1 × PBS under gravity, followed by 20 ml of 1 M NaCl and further with 10 ml of 1 × PBS. A specific IgG fraction was eluted using 100 mM glycine/HCl, pH 2.5, and sequential 0.5 ml eluate samples were collected directly into tubes containing 50 µl of 2 M Tris/HCl, pH 8 (to give a final pH of 7.6) and stored at −80 °C until required.

Expression of full-length LAT1 and LAT2 proteins in *E. coli*

Complete open reading frames (ORFs) encoding the LAT1 and LAT2 proteins were amplified from cDNA clones by PCR and cloned into the pQE-60 expression vector (Qiagen) as *NcoI*–*Bgl*II fragments. The constructs were transformed into JC201 containing pREP4 (Qiagen) which confers kanamycin resistance and expresses the *lac* repressor protein LacI. An overnight culture (10 ml) was used to inoculate 1 litre of Luria–Bertani medium containing 50 µg/ml of ampicillin and 25 µg/ml of kanamycin and growth continued at 30 °C until an A_{600} of approx. 0.5 was reached. IPTG was then added to a final concentration of 0.5 mM and induction was allowed to continue for a further 3 h. Cells were harvested by centrifugation at 5000 *g* for 20 min at 4 °C, washed in 50 ml of 100 mM Tris/HCl, pH 7.5, and stored as a cell paste at −80 °C.

Preparation of microsomes

Embryo material

L. douglasii embryos from each developmental stage were collected and stored in liquid nitrogen. Samples (1 g) were ground in a pestle and mortar under liquid nitrogen and the frozen powder added to 8 ml of extraction buffer (200 mM Hepes/NaOH, pH 7.5, 3 M NaCl, 10 mM sodium diethyldithiocarbamate, 1 mM DTT, 0.1 mM Pefabloc) and homogenized using an Ultraturrax grinder for 5 × 30 s on ice. Total homogenate samples were taken for lipid analysis and the remaining homogenate sieved through four layers of cheesecloth washed with a further 4 ml of extraction buffer. The filtrate was centrifuged at 10000 *g* for 15 min at 4 °C and the supernatant was sieved through Miracloth. The supernatant was then centrifuged at 20000 *g* for 30 min at 4 °C and again the supernatant was filtered through Miracloth. The

supernatant was further centrifuged at 100000 *g* for 2 h at 4 °C and the resulting microsomal pellets were resuspended in 500 µl of P2 buffer (100 mM Hepes, NaOH, pH 7.5, 200 mM NaCl, 5 % glycerol, 10 mM EDTA, 1 mM DTT, 0.1 mM Pefabloc) on ice. Aliquots (50 µl) were snap-frozen in liquid nitrogen and stored at −80 °C.

Leaf material

Leaf microsomes were isolated and stored essentially as described above, except that 60 g of young leaf (8 days old, 4th leaf) was homogenized in a total of 200 ml extraction buffer and the microsome preparations resuspended in 2.5 ml of P2 buffer.

E. coli

Cell pastes were resuspended in 50 ml of 50 mM Tris/HCl, pH 7.5, 0.1 mM Pefabloc for every litre of original culture. Cells were broken by two passes through a cell disrupter (Constant Systems Ltd.) at 4 °C and the resulting mixture centrifuged at 10000 *g* for 20 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 20000 *g* for 30 min at 4 °C before the final centrifugation at 100000 *g* for 2 h at 4 °C. The microsomal pellet was resuspended in 1 ml of 50 mM Tris/HCl, pH 7.5, 0.1 mM Pefabloc using a glass homogenizer on ice, and 50 µl aliquots snap-frozen in liquid nitrogen before storage at −80 °C.

Protein determination

TCA–Lowry assays [25] were used to estimate the protein concentration of all membrane preparations.

LPAAT assay

³²P-labelled oleoyl-lysophosphatidic acid (LPA) was synthesized as described previously [8] to a specific activity of 25 d.p.m./pmol. Assays were carried out in a final volume of 350 µl and contained 100 mM of Tris/HCl, pH 7.5, 0.01 % Triton X-100, 1 mg/ml BSA, 10 mM ascorbic acid, 2 mM EDTA, 100 µM [³²P]LPA and 25 µM acyl-CoA at 30 °C. The reaction was started by the addition of 30 µl of enzyme and 100 µl samples taken at 1, 3 and 5 min and then added to 720 µl of chloroform/methanol (1:1, v/v). To separate the phases, 280 µl of 1 M KCl in 0.2 M H₃PO₄ was added, and the whole mixture was vortexed for 10 s before centrifugation at 1300 *g* for 5 min at room temperature. The upper aqueous phase was discarded and the remaining organic phase dried in a rotary vacuum dryer. Dry pellets were resuspended in 40 µl of chloroform, spotted on to K6F silica gel 60 Å TLC plates (Whatman, Maidstone, U.K.) and developed in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Dry plates were exposed to a Bio-Rad Molecular Imaging Screen-BI for 4–6 h along with spots containing known dilutions of the [³²P]LPA stock. The extent of product formation was determined by comparing [³²P]LPA product spot intensity with control [³²P]LPA spot intensities. Protein concentrations in the membrane samples tested were adjusted to obtain linear reaction velocities over the course of the assay.

Staged plant material

L. douglasii plants were field-grown until they were freely flowering. Flowers were tagged the first morning they fully opened. Embryos (without seed coat) were collected at 18, 20, 22, 25 and 30 daf (days after flowering) directly into liquid nitrogen and stored until required.

Western-blot densitometry

All samples were separated on SDS/12% PAGE mini-gels (Bio-Rad Laboratories) and electro-blotted on to Hybond-ECL® (Amersham, Little Chalfont, Bucks., U.K.) nitrocellulose membrane overnight at 4 °C. Membranes were blocked by shaking for 2 h at room temperature in 50 ml blocking buffer [1 × Tris-buffered saline (TBS), pH 7.6, 5% skimmed milk powder, 0.1% Tween 20]. The membrane was incubated with 120 µl of primary antibody (affinity-purified IgG) in 50 ml antisera buffer (1 × TBS, pH 7.6, 5% skimmed milk powder, 0.01% Tween 20), unless specified otherwise, at room temperature for 1 h. Three 5 min washes in antisera buffer without antibodies was followed by incubation in 1:10000 dilution of sheep anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) diluted in 50 ml antisera buffer. The membrane was washed for four times, each time for 5 min in 100 ml of wash buffer (1 × TBS, pH 7.6, 0.05% Tween 20) and rinsed three times, each time for 1 min in deionized water. To develop the image, each membrane was soaked for 1 min in 10 ml of SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.), wrapped in Saran Wrap (clingfilm) and exposed to film for between 20 s and 1 h, depending on the intensity of the signal. Control dilutions of the relevant antigen were run alongside all samples. Exposed and developed films were scanned into the Bio-Rad Multi-Analyst software that was used to calculate the level of LAT1 or LAT2 protein in test samples relative to antigen control samples.

Lipid analysis

Samples (0.4 ml) of the total homogenate preparations from staged embryo samples were added to 6 ml of chloroform/methanol (2:1), and after mixing for 30 s, 1.6 ml of 0.88% KCl added. After phase separation, the lower phase was re-extracted with 1.5 ml of methanol/water (1:1) and transferred to a tube containing 13 µl of 50 mg/l butylated hydroxytoluene and 50 µl of 10 mg/ml 17:0 fatty acid. Solvents were evaporated in a nitrogen stream before addition of 2 ml of 1 M methanolic HCl and incubated at 80 °C for 1 h. NaCl (1 ml at 0.9 M concentration) and 2 ml of isohexane (HPLC grade) were added and the upper phase transferred to a sample vial after phase separation. The solvent was removed by evaporation, and the residue dissolved in hexane for fatty acid methyl ester analysis by GLC using a Supelco S2380 column (30 m × 0.25 mm internal diameter). Initial oven temperature was 140 °C for 2 min followed by heating to 240 °C at 4 °C/min. The injector temperature was 250 °C and the flame ionization detector temperature was 270 °C. The identified fatty-acid peaks were quantified using the internal standard as control.

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) analysis

Automatic tryptic digestion was performed on a ProGest digestion robot (Genomic Solutions Ltd., Huntingdon, Cambs., U.K.) according to the manufacturer's protocols. After digestion, 0.5 µl of each sample was spotted, together with 0.5 µl of a saturated solution of α -cyano-4-hydroxycinnamic acid matrix directly on to a MALDI target plate using an Applied Biosystems Symbiot robot. Automated MALDI analyses were carried out on an Applied Biosystems Voyager DE-STR MALDI–TOF–MS mass spectrometer with close external calibration for each sample using PE sequezyme calibration mixture I (containing des-Arg-Bradykinin, angiotensin I, Glu-fibrinopeptide B, and Neurotensin). Accumulated spectra were acquired and auto-

mated peak detection, noise reduction and peak de-isotoping were carried out using Applied Biosystems Proteomic Solutions 1 (PS1) software.

RESULTS

LAT2 can functionally replace the *E. coli* *plsC* gene

Initial attempts to replace functionally the *E. coli* LPAAT gene, *plsC*, with plant LPAAT sequences used *E. coli* strain JC231(pLEED4), provided by Dr J. Coleman (Department of Biochemistry and Molecular Biology, Medical Centre, Louisiana State University, New Orleans, LA, U.S.A.). In this strain, the *plsC* gene was deleted and replaced with a kanamycin selection marker, and LPAAT activity required for membrane lipid biosynthesis was provided by the plasmid pLEED4. This contained the *N. meningitidis* ORF D4, which encoded LPAAT [23] in a *Neisseria* spp.–*E. coli* shuttle vector selectable with erythromycin. JC231(pLEED4) was transformed with either pMAT1 or pLAT2 plasmids (which complement the *plsC*^{ts} mutant JC201) and serially sub-cultured in media containing ampicillin and no erythromycin. The aim of this was to cure pLEED4 and replace it with a plasmid encoding a plant LPAAT (Scheme 1, path A). The loss of pLEED4 was not shown, as the bacteria remained erythromycin-resistant, indicating that the *N. meningitidis* LPAAT had a strong selective advantage compared with the plant homologues and that these may not provide sufficient functionality for 100% replacement.

We therefore created the plasmid pMT149 that contained the *N. meningitidis* LPAAT gene on a plasmid with a temperature-sensitive origin of replication. This was expected to support the growth of JC231 as it contained the same LPAAT gene as pLEED4 and had the advantage that it could be cured by appropriate temperature selection in future complementation experiments. Transformation of JC231(pLEED4) with pMT149 and growth at 30 °C in the absence of erythromycin allowed the isolation of a strain, JC231(pMT149), which was tetracycline-resistant, erythromycin-sensitive and temperature-sensitive (Scheme 1, path B). The replacement of pLEED4 with pMT149 as opposed to pLAT1 or pMAT1 probably occurred because both constructs contained the same gene and there was no selective advantage in pLEED4 retention.

JC231(pMT149) was subsequently transformed, in separate experiments, with pLAT1, pLAT2, pMAT1 and pPLSC. Transformants were grown at the non-permissive temperature (42 °C) to cure them of pMT149. Complete replacement of pMT149 was achieved with pLAT2, pMAT1 (Figure 1) and pPLSC, resulting in bacteria that were tetracycline-sensitive, ampicillin-resistant and were able to grow at 42 °C. This is the first study to show that a plant LPAAT gene functionally replaces its *E. coli* equivalent. The plasmid pLAT1 was not able to replace pMT149. This negative result might be due to the inefficient translation of LAT1 and it was therefore decided to clone the ORFs from LAT1 and LAT2, together with the coconut sequence, into the expression vector pQE-60 (see below) to try and get comparable expression. Again, the expression of LAT2 (from pQE-60LAT2) enabled replacement of pMT149 (Figure 1), but plasmids pQE-60LAT1 or pQE-60Coco, containing LAT1 and coconut LPAATs respectively, were not able to support the growth of JC231.

Complementation of JC201 with plant LPAAT sequences

In addition to experiments with JC231, we tried to complement the temperature-sensitive mutant JC201 with different plant LPAATs cloned into the same expression plasmid. Several

Scheme 1 Functional replacement of *plsC* with a plant LPAAT

The two different strategies employed are shown. Path (A) shows direct replacement of pLEED4, and path (B) replacement via an intermediate. A maize LPAAT is shown in (A) and a *Limnanthes* spp. LPAAT in (B), although different LPAAT genes were used in both these experiments; see text for further details. *nla*, *Neisseria* LPA acyltransferase; Tc, tetracycline; ery, erythromycin; Amp, ampicillin.

Figure 1 Complementation of *JC231* with plant LPAATs

Plates were inoculated with an equal number of bacteria, streaked for single colonies and incubated at 30 or 42 °C. Strains were (A) *JC231*(pMT149), (B) *JC231*(pMAT1), (C) *JC231*(pQE-60LAT2) and (D) *JC231*(pLAT2). Bacteria containing pMAT1 and pLAT2 grew more slowly than the other two strains. At 42 °C, growth of *JC231*(pMT149) was visible only in the primary streak before temperature-induced loss of the plasmid, and no single colonies were seen.

Figure 2 Overexpression and purification of truncated forms of LAT1 and LAT2 proteins

(A) Total protein extracts after induction of *E. coli* BL21(DE3) (lane 1), BL21(DE3) pETLAT2 (lane 2) and BL21(DE3) pETLAT1 (lane 3). (B) Purified proteins used for raising antibodies. Lane 1, LAT2 antigen; lane 2, LAT1 antigen.

plant LPAAT sequences were cloned into the QE-60 to obtain equivalent expressions from the optimized IPTG-inducible promoter and the ribosome-binding sequence in this vector. LPAAT ORFs from *Limnanthes* spp. (LAT1 and LAT2) and coconut were used in these experiments and resulting plasmids were transformed into *JC201* and *JC201*(pREP4). The plasmid pREP4

Figure 3 MALDI-TOF-MS and peptide coverage for tryptic fragments of (A) LAT1 and (B) LAT2 antigens

Spectra were noise-filtered, peaks de-isotoped, and peak masses calibrated using tryptic autolysis peaks 842.5 and 2211.1. Underlined portions of the amino acid sequences indicate the peptide coverage for each antigen.

encoded the *LacI* repressor protein that regulated the activity of the T5 promoter in pQE-60 and should have prevented the expression of the LPAAT proteins before IPTG addition. Plasmids pQE-60Coco and pQE-60LAT1 did not complement either of the strains and allow growth at 42 °C in the presence or absence of IPTG. It is somewhat surprising that the very similar proteins LAT1 and LAT2 differ in their ability to complement *plsC* mutations in *E. coli*. Complementation of the temperature-sensitive phenotype of JC201 with pQE-60LAT2 was only possible in bacteria containing pREP4 and on plates containing no IPTG. This suggests that substantial expression of LAT2 is toxic to JC201 and the absence of pREP4 or the addition of too much IPTG causes the amount of LAT2 expressed to be lethal.

Expression of full-length and truncated forms of LAT1 and LAT2

Neither of the full-length proteins LAT1 or LAT2 could be expressed in pET (T7 promoter system) or QE-60 (T5 promoter system) vectors to allow the purification of sufficient antigen for immunization purposes, although both were subsequently detected immunologically at low levels (results not shown). The low amounts of protein were probably due to either direct toxicity to the host cell or limited capacity of the *E. coli* membrane system to accommodate membrane proteins [14]. It was therefore decided to express truncated proteins, lacking membrane-spanning domains. This strategy would produce large proteins that might be more advantageous than small peptides for use as antigens. Computer predictions for each protein (DNA StriderTM1.2) identified hydrophobic, putative membrane-spanning domains between residues 1–70 and 305–355 in LAT1 and residues 30–93 in LAT2. Truncated LAT1 and LAT2 antigens were obtained by expressing the more hydrophilic regions of the proteins in the expression vector pET-11d. The antigen for LAT1 was composed of amino acids 81–304 of the full-length protein (377 amino acids) and the antigen for LAT2 was composed of amino acids 97–281 of the full-length protein (281 amino acids). Expression of these proteins appeared as dominant species on Coomassie Blue-stained gels of *E. coli* extracts (Figure 2A). In each case, the majority of the expressed protein was in inclusion bodies that were washed in high-salt buffer, solubilized by boiling in SDS and proteins purified using a Bio-Rad preparative SDS/PAGE-Prep-Cell (Figure 2B). The identity of the purified proteins was confirmed using MALDI-TOF peptide mass ‘fingerprinting’ after excision from gels and proteolysis (Figure 3).

Antibodies raised against recombinant truncated LAT1 and LAT2 do not cross-react

Antibodies against LAT1 and LAT2 were raised and purified as described in the Experimental section. The anti-sera were used at a dilution of 1:5000 indicating that they were of high avidity. It was clear that these anti-sera were specific for their respective antigens and did not cross-react with the other truncated acyltransferase protein (Figure 4). Any detection of proteins in plant tissues on Western blots by these anti-sera was therefore not due to cross-reactivity between LAT1 and LAT2 proteins. The detection limits of the affinity-purified anti-sera on Western blots were 2.5 ng for the LAT1 antigen and 1 ng for the LAT2 antigen.

Patterns of expression of LAT1 and LAT2 differ in leaf and seed material

The polyclonal anti-sera produced were affinity-purified to reduce cross-reactivity with non-LPAAT proteins in crude plant

Figure 4 Western-blot analysis of purified LAT1 (lanes 1) and LAT2 (lanes 2) antigens using (A) affinity-purified LAT1 antibody and (B) affinity-purified LAT2 antibody

LAT1 (10 ng) and of LAT2 (2.5 ng) antigens were loaded on to the protein gels.

extracts. This was found to be essential to get a clean background on Western blots. The distribution of LAT1 and LAT2 in both microsomal and 100000 *g* supernatant fractions from mid-stage developing embryos and 8-day-old leaf material was examined by Western-blot analysis using the purified anti-sera.

LAT1 was detected in the microsomal fraction from leaf after exposing the blot for 15 min (Figure 5A), but was not detected in the 100000 *g* supernatant even after exposure of the blot for several hours. LAT1 was detected in embryo microsome fractions, but only after over-loading of the gel and allowing long exposure times (see below). The anti-LAT1 antibody consistently detected two bands in *Limnanthes* spp. leaf material. The molecular mass of the major band was approx. 36 kDa, whereas that of the much weaker secondary band (< 5% of the total) was approx. 28 kDa. Probably, the smaller secondary band represented a degradation product of LAT1 produced during the 3–4 h preparation of microsomes from the whole tissue. LAT2 was detected in the embryo microsomal fraction (Figure 5B), but not in the equivalent 100000 *g* supernatant or leaf microsomal fractions even after several hours of exposure.

The observed molecular mass for both LAT1 and LAT2 detected in plant material were approx. 7–8 kDa smaller than the values predicted using amino-acid sequences. LAT1 and LAT2 had predicted molecular mass of 42.7 and 31.6 kDa, and observed mass of 36 and 23.5 kDa respectively. Anomalous migration in SDS/PAGE is not an unusual phenomenon. In this case, it was thought to be due to the hydrophobicity of LAT1 and LAT2, since they are integral membrane proteins.

Quantification of levels of LAT1 and LAT2 in *Limnanthes* spp. leaf and developing seed

Pure antigen standards were used to quantify the amount of LAT1 and LAT2 in various plant tissues using Western-blot analysis. A quantification standard of pure antigen was included in the main gel and an additional gel containing a standard

Figure 5 Immuno-detection of LAT1 and LAT2 proteins in microsomal fractions using affinity-purified anti-sera against (A) LAT1 and (B) LAT2

Samples for blot (A) were (1) 100 μ g of leaf microsomal protein (Lm), (2) 100 μ g of leaf 100000 g supernatant protein (Ls), (3) 100 μ g embryo microsomal protein (Em), and the blot was exposed for 15 min. Samples for blot (B) were (1) 5 μ g of embryo microsomal protein (Em), (2) 25 μ g of embryo 100000 g supernatant protein (Es), (3) 25 μ g of leaf microsomal protein (Lm), and the blot was exposed for 1 min.

dilution series of the antigen was run at the same time for comparison. All were analysed by densitometry, and for each antigen dilution series, the measured volume (average pixel density multiplied by area) was proportional to the amount of antigen loaded on to the gel. By using an optimum load for gels, together with appropriate exposure times and standard curves, it was possible to quantify the amounts of both LAT1 and LAT2 in terms of pg/ μ g membrane protein.

LAT2 was only detected in embryos that were 22 daf or older, even with long exposures of blots made from gels loaded with five times the amount of protein for the 18 and 20 daf stages (Figure 6). It was clear that LAT2 was expressed at a defined developmental stage in the embryo between 20 and 22 daf. The level of expression was considerably higher than LAT1 in both leaf and embryo, and reached a peak at 305 pg/ μ g membrane

Figure 6 Western-blot analysis of total embryo protein extracts with affinity-purified LAT2 antibody

Numbers on the top denote daf of the embryos used for protein preparation and 50 μ g (18 and 20 daf) or 10 μ g (22, 25, 30 daf) of the protein was loaded on to the gel.

Figure 7 Quantification of LAT1 and LAT2 antigens

The amount of LAT1 in leaf microsomes and LAT2 in embryo microsomes.

protein in the 25 daf stage before decreasing (Figure 7). It should be noted that for quantification purposes (Figure 7) short exposures of 1–15 min were used, but longer exposures of up to 24 h were used to show the lack of LAT2 protein in early-stage membrane preparations (Figure 6).

LAT1 was not detected in total protein preparations from young *Limnanthes* spp. leaves but was detected in membrane preparations from both leaves (Figure 5A) and embryos. The level of LAT1 expression in leaf membranes was determined as 27 pg/ μ g membrane protein (Figure 7). In embryo microsomes LAT1 concentration was low but in heavily loaded gels of all embryo stages tested, LAT1 was detected as a faint band against a high background after overnight exposure of blot. Since the sensitivity of the antibodies used was known, it was possible to estimate the upper limit of LAT1 concentration in embryo samples. The maximum LAT1 level in embryo membranes was 12.5 pg/ μ g membrane protein and was similar at all developmental stages. This value was based on a faint signal being detected with a loading of 200 μ g protein and a detection level of 2.5 ng LAT1. We conclude that LAT1 is a very low abundance protein that is present in both tissues and at all developmental stages of the embryo.

Lipid analysis of developing *L. douglasii* embryos

Very-long-chain unsaturated fatty acid accumulation in the developing embryos (Figure 8) temporally coincided with LAT2 expression and an increase in the total fatty acid content. LAT2 expression began between 20 and 22 daf and 22:1 accumulation occurred at its maximal rate in the same time frame. The accumulation of 22:1 Δ 13 in developing embryos reflected the increase in total fatty acid that occurred over a relatively short period of time (20–25 daf). The proportion of both 18:1 and 16:0 in total fatty acids decreased from 20 daf, reflecting the different fatty acid compositions in TAGs as opposed to membrane lipids.

Acyl group selectivity of LAT1 and LAT2

To evaluate the substrate selectivity of LAT1 and LAT2, the two proteins were expressed in *E. coli* and microsomes from these bacteria used in LPAAT assays. Genes encoding full-length

Figure 8 Lipid analysis of developing *L. douglasii* embryos

(A) (●—●), proportion of 16:0; (▲—▲), 18:1 Δ9; (■—■), 22:1 Δ13; (◆—◆), 22:2 Δ5, 13; (×—×), 20:1 Δ5; fatty acids during embryo development. The total fatty acid content (B) and 22:1 Δ13 content (C) per embryo are also shown.

Figure 9 Acyl-CoA utilization by membrane fractions from *E. coli* expressing LAT1 and LAT2

(A) Initial reaction rates for PA formation with 18:1 acyl-CoA and 18:1-LPA by membrane fractions from: (●—●), JC201(pREP4) pQE-60LAT2; (◆—◆), JC201(pREP4) pQE-60LAT1; (×—×), JC201(pREP4) pQE-60 (vector alone); (▲—▲), XL1-Blue. The assay with XL1-Blue contained 10 µg of membrane protein and the others contained 100 µg of membrane protein. (B) Ratio of reaction rates for 12:0, 16:0, 18:1 Δ9, and 22:1 Δ13 acyl-CoAs. The rate for 18:1 acyl-CoA is designated 1. The range is shown from duplicate assays for LAT2 and the standard error from three assays for XL1-Blue.

LAT1 and LAT2 were inserted into the plasmid pQE-60 and the proteins expressed in the temperature-sensitive mutant host JC201(pREP4) at 30 °C. *E. coli* strain XL1-Blue was used as a source of membranes for wild-type *E. coli* LPAAT selectivity in these assays. Microsomes prepared from these bacteria were used in LPAAT assays with labelled oleoyl-LPA and different acyl-CoA donors. The activity of the JC201 *plsC* gene product was very low and hence can be subtracted from the activity values for acyltransferases expressed in this strain [12]. Reaction rates obtained with various membrane fractions using 18:1 acyl-CoA as a donor are shown (Figure 9A). It should be noted that the activity seen with membranes from XL1-Blue was obtained with 10-fold less protein added. Nevertheless, the activity in membranes from bacteria expressing LAT1 or LAT2 was clearly higher than that detected in JC201(pREP4) pQE-60 alone.

The incorporation of fatty acids into 18:1-LPA from different acyl-CoAs was measured with the different membrane fractions (Figure 9B). The wild-type *E. coli* LPAAT, PLSC (shown by XL1-Blue microsomes), preferred 18:1-CoA as substrate, although it used 12:0-CoA and 16:0-CoA at a significant rate. The substrate utilization profiles for LAT1 and LAT2 are very different from that of PLSC. This strongly indicates that the LPAAT activity measured in these membranes is due to the plant enzyme and not due to the reversion of *E. coli* mutant host JC201. The substrate selectivity profiles for LAT1 and LAT2 clearly reflect the different biological roles of the two enzymes *in vivo*. LAT2 is the least selective, and also showed significant 22:1-CoA activity, whereas LAT1 had no detectable 22:1 activity. This reflects the incorporation of 22:1 Δ13 at the *sn*-2 position in *L. douglasii* seed storage oil. LAT2 also used saturated acyl-CoAs and may be useful in increasing the proportion of such saturated fatty acids in seed material, and hence be important from a biotechnological standpoint. LAT1 very strongly selects against saturated and long-chain unsaturated acyl-CoAs and is only able to use oleoyl-CoA from the substrates. This might be due to its possible role within the plant as an LPAAT responsible for membrane production in the endo-

plasmic reticulum. Predominantly, plant cellular membranes are composed of C₁₆/C₁₈ unsaturated lipids and uncontrolled incorporation of saturated or longer chain fatty acid moieties would severely affect the membrane function [26].

DISCUSSION

The aim of this work was to characterize the two different *Limnanthes* spp. LPAATs, LAT1 and LAT2 with regard to their substrate selectivity and to determine their localization in different plant tissues using antibodies. For selectivity studies we attempted to express both proteins in *E. coli* in order to use isolated microsomal fractions for assays. Although the activity of the mutated LPAAT in JC201 is low [21], we preferred to express LAT1 and LAT2 in strain JC231 with a disrupted LPAAT gene, so the detected enzyme activity would be solely from the plant protein. This was possible only with plasmids pMAT1 and pLAT2 (that were previously shown to complement JC201) and pQE-60LAT2. To our knowledge, this is the first evidence that a plant LPAAT gene can completely replace the function of its bacterial counterpart. The failure of pQE-60LAT1 to complement JC231 was surprising, given that it encodes an acyltransferase very similar to that in pMAT1 and contained the same vector as pQE-60LAT2. Complementation experiments were extended to include a coconut clone and *E. coli* strains JC201 and JC201(pREP4), with selection at 42 °C in the presence of different amounts of IPTG. The role of pREP4 was to minimize the potential toxic effects of the unregulated leaky expression of membrane-bound enzymes. Again, LAT1 did not complement the LPAAT mutants and LAT2 could only do so with the repressor plasmid present and without IPTG. This indicated that expression of LAT2 above a certain level was toxic, and suggested that the isolation of pLAT2 by complementation of JC201 [8] was due to inefficient translation of the protein from a fortuitous translation initiation signal.

For complementation of JC201 or functional replacement of the bacterial gene in JC231 by a plant LPAAT, it had to (a) be expressed at a sufficient but not toxic level, (b) fold correctly and assemble into the correct metabolic compartment and (c) have suitable substrates available for catalysis. Clearly, this was achieved with LAT2 and MAT1 but not with LAT1 or the coconut LPAAT, despite the use of similar constructs under the same selection conditions. It may be the substrate selectivity of the enzyme encoded by pQE-60Coco prevented the complementation or functional replacement of *E. coli* LPAAT, since the coconut enzyme has a strong preference for 12:0-CoA [9]. However, it is difficult to explain why LAT1 did not complement, even when expressed from constructs including the strong lipoprotein translation initiation sequence. Other reasons for non-complementation include insufficient expression or activity level, targeting to the outer membrane or insertion into the *E. coli* cytoplasmic membrane in the wrong orientation. Although complementation of *E. coli* mutants is a powerful method for cloning new LPAAT genes, lack of complementation does not imply the absence of an LPAAT cDNA in a library or the lack of biological activity in a protein encoded by an individual clone.

This proved to be the case for the LAT1 protein, as pQE-60LAT1 was not able to support growth at 42 °C, but microsomes from bacteria grown at 30 °C had substantially more LPAAT activity than those from the host strain, and in particular a completely different acyl-CoA selectivity. The use of microsomes from JC201 containing plant acyltransferase LAT1 grown at 30 °C enabled the substrate selectivity of LAT1 to be assessed for the first time and the selectivity of LAT1 and LAT2 to be

compared. LAT1 appeared to be a highly selective acyltransferase that could only use 18:1-CoA out of the substrates tested. The inability to incorporate saturated or long-chain fatty acids was consistent with its proposed role in membrane, as opposed to storage lipid biosynthesis. LAT2 was much less selective than LAT1 and readily utilizes both 16:0-CoA and 22:1-CoA. The higher 22:1-CoA incorporation compared with 18:1-CoA is in agreement with the results described previously [12] and it is also interesting that LAT2 can use 12:0 as a substrate whereas LAT1 cannot. The substrate selectivity of LAT2 was not reflected in the acyl chain length profile at the *sn*-2 position of *Limnanthes* spp. seed storage oil, as the proportion of 16:0 or 18:1 at this position is approx. 5% [10]. These fatty acids were apparently excluded from the storage oil by substrate availability rather than enzyme selectivity.

The overexpression of full-length membrane-bound enzymes, such as LAT1 and LAT2, for antibody generation is problematic. This work has shown that the use of antigens based on non-membrane portions of the proteins enables the production of specific and sensitive anti-sera to technically demanding hydrophobic proteins. Specific and sensitive anti-sera are essential tools for protein quantification and future immuno-localization studies within the plant cell. Using these antibodies in Western-blot analysis, both LAT1 and LAT2 have observed molecular masses 7–8 kDa smaller than predicted values. This is similar to the disparity between the observed and predicted molecular mass for the *E. coli* GPAT protein PlsB, which has a predicted molecular mass of 92 kDa but an observed molecular mass, in several studies, of 83 kDa [27]. There are two reasons why membrane proteins could have lower observed sizes on SDS/PAGE gels than predicted [28]. First they were not completely dissociated by boiling in SDS loading buffer although it was a rarely reported phenomenon unlikely to occur in two independent and dissimilar proteins such as LAT1 and LAT2. The second and more plausible reason is that they are unusually hydrophobic or contain large hydrophobic domains that bind more SDS than random amino-acid sequences; this probably increased their mobility in SDS/PAGE, reducing the apparent molecular mass. This is supported by the observation that in both cases the antigens, made by cutting off the postulated membrane-spanning hydrophobic domains, resolve in SDS/PAGE according to the predicted masses.

To our knowledge this is the first quantification of membrane-bound acyltransferases in plant tissue. Neither LAT1 nor LAT2 are highly expressed proteins as they comprise a maximum of 0.0027 and 0.031% of the total membrane protein in leaf and seed respectively. The induction of LAT2 to levels greater than 20-fold compared with that of LAT1 in the seed coincides with the major phase of TAG biosynthesis. Having shown that the antibodies raised against the two LPAATs from *Limnanthes* spp. are both specific and sensitive, we now have a reagent for following the expression of these proteins in transgenic plants and for immuno-localization studies within *L. douglasii*. They may be able to be used to identify other proteins that associate with LPAATs in plant membranes and gain a greater insight into compartmentalization of lipid biosynthesis and protein–protein interactions within plant cells.

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