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Identification of a cDNA that encodes a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase from *Limnanthes douglasii*

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Abstract

Two different techniques were used to isolate potential cDNAs for acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (LPA-AT) enzymes from *Limnanthes douglasii*. Both heterologous screening with the maize pMAT1 clone and *in vivo* complementation of the *Escherichia coli* mutant JC201 which is deficient in LPA-AT activity, were carried out. Clones identified by these procedures were different. Homology searches demonstrated that the clone isolated by heterologous probing, pLAT1, encodes a protein which is most similar to the maize (open reading frame in pMAT1) and yeast SLC1 proteins, which are putative LPA-AT sequences. This *L. douglasii* sequence shows much lower homology to the *E. coli* LPA-AT protein PlsC, which is the only LPA-AT sequence confirmed by over-expression studies. The clone isolated by complementation, pLAT2, encodes a protein with homology to both SLC1 and PlsC. It was not possible to over-express the complementing protein encoded by pLAT2 but further experimentation on membranes from complemented JC201 demonstrated that they possess a substrate specificity distinctly different from PlsC and similar to *Limnanthes* sp. microsome specificity. This data strongly supports the contention that pLAT2 is an LPA-AT clone. Northern blot analysis revealed different expression patterns for the two genes in pLAT1 and pLAT2. Transcription of the gene encoding the insert of pLAT2 occurred almost exclusively in developing seed tissue, whilst the cDNA of pLAT1 hybridised to poly(A)$^+$ mRNA from seed, stem and leaf, demonstrating more widespread expression throughout the plant. Southern blot analysis indicated that the cDNA of pLAT2 was transcribed from a single-copy gene while that for pLAT1 was a member of a small gene family.

Introduction

Modification of naturally occurring triacylglycerols (TAGs) in oilseed crops to provide industrially useful products is currently of great interest. One aim is the development of a *Brassica napus* cultivar which is able to incorporate very high levels of erucic acid (22:1 Δ13) into its seed oil and preferably synthesize large amounts of trierucin (trierucoglycerol) [25]. Currently available

The nucleotide sequence data reported will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession numbers Z46836 (pLAT2) and Z48730 (pLAT1).

high-erucic acid cultivars of *B. napus* are the product of selective breeding programmes and contain a maximum of 55% erucic acid in their seed oils. In common with almost all other members of the Brassicaceae, these cultivars are unable to incorporate erucic acid at the *sn*-2 position of the TAGs [25].

The composition of a seed oil TAG depends on both the relative pool sizes of acyl-CoA thioesters and the acyl-CoA selectivity of three membrane-bound acyltransferase enzymes required for its synthesis [3]. It is apparent that the exclusion of erucic acid from the *sn*-2 position in *B. napus* TAGs is a result of strong discrimination against erucoyl-CoA by the acyl-CoA:1-acyl-*sn*-glycerol-3-phosphate acyltransferase (LPA-AT) enzyme [1, 5]. Transfer to *B. napus* of a gene encoding an LPA-AT which can utilise erucoyl-CoA is a potential way of overcoming the theoretical limit of 66% erucic acid in *B. napus* oil, determined by the selectivity of the native LPA-AT. Microsomes from seeds of members of the genus *Limnanthes* are able to synthesize dierucoyl-phosphatidic acid [5, 18] and hence LPA-AT genes from these would be good candidates for the above approach. Unfortunately, membrane-bound LPA-AT enzymes have proved difficult to purify and to date no amino acid sequence data derived directly from protein sequencing, allowing isolation of the gene, has been reported for any plant LPA-AT.

An alternative approach is complementation cloning, in which cDNAs are isolated by their ability to correct a selectable phenotype in *Escherichia coli* [8]. We have used this technique to attempt to identify cDNAs encoding plant LPA-AT enzymes by complementation of the temperature sensitive phenotype of the *E. coli* strain JC201 [7]. This strain has no detectable LPA-AT activity, even at the permissive growth temperature of 30 °C, and has been used previously to identify the *E. coli* LPA-AT gene (*plsC*) by complementation [6].

We recently published data obtained after complementation experiments using a maize endosperm cDNA library [2]. The complementing cDNA clone (pMAT1) restored LPA-AT activity to JC201 membranes and the protein it encodes showed most similarity to the yeast protein SLC1, thought to be a eukaryotic LPA-AT [20]: the *E. coli* LPA-AT protein PlsC was not in the top 40 matching proteins. Further sequence homology was found in conserved blocks of amino acids in PlsC, SLC1 and the maize protein, indicating the likelihood that pMAT1 encodes an LPA-AT enzyme. Since *Limnanthes douglasii* contains an LPA-AT of potential biological interest, we have attempted to isolate similar clones from a developing seed cDNA library using both heterologous screening and complementation cloning techniques.

This paper describes the results of such experiments during which two different cDNA clones have been isolated and characterised. One cDNA is highly homologous to the previously reported maize clone [2] but the other encodes a protein which has no significant homology to any plant protein sequence. Instead the translated sequence is homologous to published LPA-AT sequences from bacteria and yeast: membranes isolated from JC201 containing this new cDNA have an altered specificity for erucoyl CoA in LPA-AT assays. We believe that this new cDNA is an LPA-AT gene from *L. douglasii* and the sequence reported here may be of use to genetically manipulate *B. napus* to synthesise trierucin.

Materials and methods

Materials

Limnanthes douglasii plants were greenhouse-grown and seeds of stages III and IV, as defined previously for *Limnanthes alba* [16], collected. After removal of the seed coat, embryos were immediately frozen in liquid nitrogen. Leaf and stem material was also collected and frozen in liquid nitrogen. All chemicals. with the exception of those listed below, were from Sigma. Yeast extract and tryptone used in LB medium were obtained from Oxoid and Becton Dickinson, respectively, and agar was purchased from Merck. Oligo (dT)-cellulose was purchased from Collabo-

rative Biomedical Products. [γ-^{32}P]-dATP (30Ci/mmol) [α-^{32}P]-dCTP (400Ci/mmol) and Hybond N were from Amersham International. Silica thin-layer chromatography plates (type K6F) were made by Whatman and the liquid scintillant Ecoscint A was obtained from National Diagnostics.

Bacterial strains, growth media and transformation

XL1-blue, which was used for construction and maintenance of the *Limnanthes* cDNA library in λZAPII, plasmid rescue and all routine DNA manipulations, has the genotype F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 supE44lac* [4]. The mutant used for isolation of *Limnanthes* cDNAs was JC201, which is *plsC thr-1 ara-14 (gal-attλ)*-99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1 [6]. Bacteria were grown on LB medium [23] containing 15 μg/ml tetracycline, 50 μg/ml ampicillin and 100 μg/ml streptomycin where required. Transformation of *E. coli* was done by electroporation as previously described [2].

Construction of cDNA library

RNA was isolated from *L. douglasii* embryos using hot-SDS [11] and poly(A)$^+$ mRNA purified with oligo (dT)-cellulose spun columns as described for the mRNA purification kit from Pharmacia Biotech. A cDNA synthesis kit (Pharmacia Biotech) was used to make cDNA from 5 μg of mRNA, with first strand synthesis primed with oligo (dT). The cDNA was size-selected with a Sephacryl S-300 spun column and ligated to *Eco* RI adaptors before ligation into *Eco* RI-cut, dephosphorylated λZAPII arms (Stratagene). *In-vitro* packaging was performed using the Gigapack II Gold system (Stratagene). The quality and integrity of the library was checked by screening with *B. napus* enoyl reductase [14] probe, which hybridised to a clone including the full coding region of the *L. douglasii* homologue. A plasmid cDNA library was made from an unamplified sample of the λZAPII library by *in vivo* excision using the helper phage R408 (Stratagene) following protocols detailed in Delauney and Verma [8]. During plasmid rescue, ca. 1×10^6 colonies were scraped into LB medium, pooled and grown for a further 3 h before final plasmid preparation.

DNA manipulations and sequencing

All DNA manipulations and subcloning were carried out using standard protocols [23]. Sequencing was carried out using an Applied Biosystems 373A DNA sequencer (Durham University sequencing service, Ms J. Bartley). Computer analysis of DNA sequence was performed using DNA Strider [19], and the SEQNET facility at the SERC computing facility at Daresbury, UK, which includes the Wisconsin Package [22].

Northern/Southern blot analysis

For northern blot analysis poly(A)$^+$ mRNA from developing embryo, leaf and stem (isolated from stem and leaf as described above) was separated by electrophoresis through a 1.5% formaldehyde/MOPS agarose gel [23] and transferred to Hybond N by capillary blotting with 20 × SSC. Two identical blots were made with samples run on the same gel and transferred to the same filter, which was then cut in half. Hybridisation to ^{32}P-labelled DNA probes was performed at 42 °C in 50% formamide, 2 × Denhardt's solution, 0.1% SDS, 200 μg/ml denatured herring sperm DNA and 5 × SSPE. For both northern and Southern hybridisation, ^{32}P-labelled DNA probes were made by random priming [10] using a Megaprime labelling kit from Amersham. For Southern blot analysis, *L. douglasii* chromosomal DNA was isolated from leaves using CTAB extraction buffer [9]. DNA samples were digested with various enzymes, separated on a 0.7% agarose gel and transferred to Hybond N membrane according to the manufacturer's instructions. Hybridisation

was at 60 °C in 6 × SSC, 1x Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate and 1 mM EDTA after pre-hybridisation in the same solution minus EDTA and plus 50 μg/ml denatured herring sperm DNA.

*Synthesis of [^{32}P]-1-erucoyl-*sn*-glycerol-3-phosphate*

^{32}P-labelled 1-erucoyl-*sn*-glycerol-3-phosphate (22:1-LPA) was made in a linked enzymatic synthesis from [γ-^{32}P]-dATP, glycerol and erucoyl-CoA. The enzymes used were glycerokinase and purified plastidial glycerol-3-phosphate acyltransferase from *Arabidopsis thaliana* which had been over-expressed from a pET-3c construct [21] and purified to > 95% with two anion exchange chromatographic steps on Q-sepharose (Pharmacia Biotech). The synthesis mixture consisted of 250 mM HEPES/NaOH pH 7.4, 10 mM glycerol, 2.5 mM $MgSO_4$ 0.5 mM ATP, 333 nM [γ-^{32}P]-dATP, 100 U *E. coli* glycerokinase (1 U converts 1.0 μmol substrate per minute), 10 mg/ml BSA, 2 mM erucoyl-CoA and a 1:20 dilution of pooled active fractions from the glycerol-3-phosphate acyltransferase chromatography. A 5 ml reaction was incubated for 16 h at 25 °C. Two volumes of methanol and one volume of chloroform were added with mixing and after 5 min at room temperature, a further 5 ml of chloroform and 5 ml of 0.2 M H_3PO_4 in 1 M KCl were added. After mixing and resolution of the phases by centrifugation, the chloroform layer (10 ml) was removed and reduced to 0.5 ml by vacuum centrifugation. This was applied to a thin-layer chromatography (TLC) plate, which was developed with chloroform/methanol/water (12:6:1); the labelled compounds were localised by autoradiography. The [^{32}P]-22:1-LPA (Rf 0.15) was extracted from the silica with methanol, which was then removed completely by vacuum centrifugation before the product was resuspended in 0.2% octyl-β-*D*-glucopyranoside to a final concentration of 1 mM.

Preparation of membranes

E. coli membrane fractions were prepared as described [2]. Microsomes from developing seeds of high-erucic acid rape (cv. Miranda) were prepared as follows, with all steps carried out at 4 °C. Seed material (10 g) aged 4–5 weeks after flowering was ground and 25 ml homogenisation buffer (10 mM sodium phosphate pH 6.2, 4 mM EDTA, 1 mM DTT) added. Cells were homogenised on ice/water with a Polytron for 2 min in 20 s bursts with time allowed for the sample to cool to less than 4 °C in between each one. The sample was filtered through four layers of muslin and cleared by centrifugation at 40 000 *g* for 30 min. The supernatant was centrifuged at 200 000 *g* for 30 min and the pelleted microsomes resuspended in homogenisation buffer with 0.5 M NaCl added. The salt-washed microsomes were then collected by centrifugation at 200 000 *g* for 30 min and resuspended in 1.25 ml homogenisation buffer before snap-freezing in liquid nitrogen and storage at −80 °C.

1-acyl-G-3-P-acyltransferase assays

Assay mixtures consisted of 100 mM Tris-HCl pH 9.0, 0.5 mM $MgCl_2$, 0.01% Triton X-100, 1 mg/ml BSA. 0.2% octyl-β-*D*-glucopyranoside, 1 mM sodium acetate pH 6.0, 100 μM [^{32}P]-22:1-LPA and 100 μM oleoyl-CoA or erucoyl-CoA. Assays were started by addition of membrane fractions and 100 μl samples were removed and mixed with 2.5 ml chloroform/methanol (1:1) before storage on ice to stop the reaction. After the addition of 1 ml of 0.2 M H_3PO_4 in 1 M KCl and vigorous mixing, the phases were separated by centrifugation. The lower glycerolipid-containing chloroform layers were removed and reduced to 100 μl by vacuum centrifugation before application to TLC plates. These were developed using the same solvent system described above and labelled compounds visualised by autoradiography. Phosphatidic acid spots (Rf 0.3) were scraped into 1.5 ml methanol and [^{32}P]-22:1-LPA incorporation determined by liquid scintillation counting after mixing and addition of 4 ml Ecoscint A.

Results

Heterologous screening of cDNA library

The *L. douglasii* seed cDNA library in the vector λZAPII was screened using standard protocols [23] with a 600 bp probe corresponding to the protein sequence from R-107 to A-287 in the putative LPA-AT encoded by pMAT1 [2]. A clone, designated pLAT1, was isolated and the sequence of its cDNA insert determined. The 1.5 kb cDNA contained only one long ORF of 377 amino acids encoding a protein of relative molecular mass 42724. This protein was 82.1% similar and 66.8% identical to the maize protein sequence previously described [2]. A stop codon present before the first methionine of the ORF indicates that pLAT1 includes the full coding region of the gene and, since homologies exist right at the N-termini of the two proteins, the putative translation start suggested for the maize protein [2] is likely to be correct. Computer analysis [24] of the OWL database in a search for homologous proteins showed that the ORF in pLAT1 was homologous to the putative yeast LPA-ATSLC1 (21.5% identity over 251 amino acids) but not PlsC, the *E. coli* LPA-AT.

Complementation of JC201

L. douglasii developing seed cDNA plasmid clones were selected for their ability to complement the temperature-sensitive phenotype of JC201 bacteria and restore growth at 44 °C. Complementation experiments were carried out as described previously [2] except that the library had not been split into fractions according to insert size during construction and only one cDNA sample was used for the original transformation. DNA was isolated from all transformed colonies that grew at 44 °C and used for re-transformation of JC201. About 6000 colonies grew at the non-permissive growth temperature after this second transformation, compared to 11 colonies for JC201 samples transformed with the plasmid vector (pBS SK$^+$) alone. Plasmid DNA was isolated from 18 phenotypically complemented colonies from the second transformation. All contained cDNA inserts of the same size and one of the plasmids, designated pLAT2, was used in further studies.

Analysis of pLAT2

The cDNA insert of pLAT2 was sequenced in both directions with reactions primed with standard M13 forward and reverse primers using digested and re-ligated subclones of pLAT2 as templates. The cDNA is 1068 bp long followed by a short poly(A) tail of 7 bp. A consensus polyadenylation signal sequence AATAAA is located 60 bp upstream from the poly(A) tail. Translation of the cDNA sequence (Fig. 1) revealed the presence of two large open reading frames (ORFs) which are both in the 5′ to 3′ direction of the cDNA and could be translated as the products of transcription from the *lacZ* promoter of pBluescript SK$^-$. Neither of the ORFs is in frame with *lacZ* which would be translated to produce a truncated fusion protein terminating at the TAA codon indicated (Fig. 2). It is likely that in *E. coli* the complementing polypeptide is initiated at Met-35 which has a purine-rich sequence upstream that could initiate translation. The ORF in frame 3 of pLAT2 has no putative Shine-Dalgarno sequence preceding its first methionine

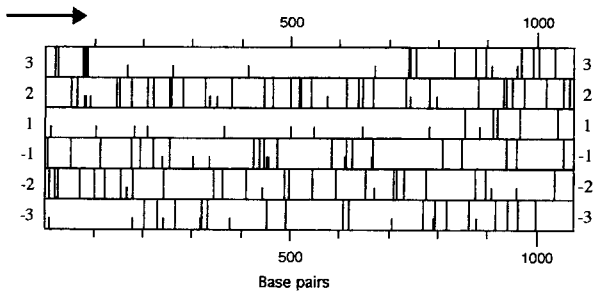

Fig. 1. Map of the open reading frames in the cDNA insert of pLAT2. The schematic diagram showing open reading frames in all six phases was derived from the cDNA sequence using DNA Strider [19]. Full vertical lines represent stop codons and half vertical lines ATG sequences. The arrow shows the direction of transcription from the *lacZ* promoter in pBS SK$^-$.

Fig. 2. Sequence of the 5′ end of the cDNA of pLAT2. The figure shows part of the sequence of pLAT2 with a translation of all three reading frames underneath. Numbers on the left refer to open reading frames as in Fig. 1. The stop codon responsible for truncation of the *lacZ* gene product is shown in bold and underlined and Met indicate possible start methionines for the large open reading frames downstream of this. A purine rich region upstream of one of the methionines is underlined. * indicate stop codons in the translations and M represents the first methionine of the largest ORF in the cDNA of pLAT2. Amino acid residue numbers are shown above the sequence in italics.

(Met-56) and a derivative of pLAT2 in which this ORF was put in frame with the *lacZ* gene (from residue 35: -G-I-A-I-V- in Fig. 2) failed to complement JC201 cells. This data strongly suggests that the 281 amino acid open reading frame in the cDNA of pLAT2 (in frame 1, Fig. 1) encodes the complementing protein which in JC201 is translated as a truncated product of 250 residues.

Sequence homology determination

The sequences of both large ORFs in pLAT2 were used in computer analysis [24] of the OWL database for homologous proteins. The smaller protein sequence, corresponding to frame 3 in Fig. 1, showed no significant homology to any previously reported protein. The 281 amino acid ORF was most homologous to previously reported LPA-AT sequences, with the top match (optimised score 283, 27.9% identity over 244 residues) being PlsC. An even greater degree of identity over a smaller region (35.3% over 187 residues; optimised score 279) was present with the SLC1 protein of yeast, which is thought to be the first eukaryotic LPA-AT isolated [20]. Alignment of the largest ORF in pLAT2 with PlsC using the FASTA algorithm [17] is shown in Fig. 3. Blocks of identical residues exist throughout the overlapping regions with stretches of conserved residues between these blocks. Using the Smith and Waterman algorithm [24] the *Limnanthes* protein overall is 50.6% similar to PlsC, but for the 141 residue stretch of overlap from Ala-67 in this protein the similarity score is 55.4% with 38% identity. The hydrophobicity plots of these two proteins are similar except for an additional 30 amino acid hydrophilic region at the N-terminal end of the protein encoded by pLAT2.

Similar results are obtained if the large ORF in pLAT2 is aligned with SLC1, although some identical matching residues are different to those in the PlsC alignment. Part of the alignment of all three of these sequences is shown in Fig. 4. Of the 180 amino acids in the pLAT2 translation, 35 are identical to residues found in the *E. coli* and putative yeast LPA-AT sequences. The large ORFs in pLAT1 and pLAT2 both encode proteins with similarities to LPA-AT sequences and several blocks of a few amino acids are present in both translated sequences. Although some residues are conserved between the two proteins and overall they are 52% similar and 27.6% identical, the distinct difference between them is highlighted when they are compared to PlsC, which is not in the top 40 matches for the ORF in pLAT1 but is the top match for that in pLAT2.

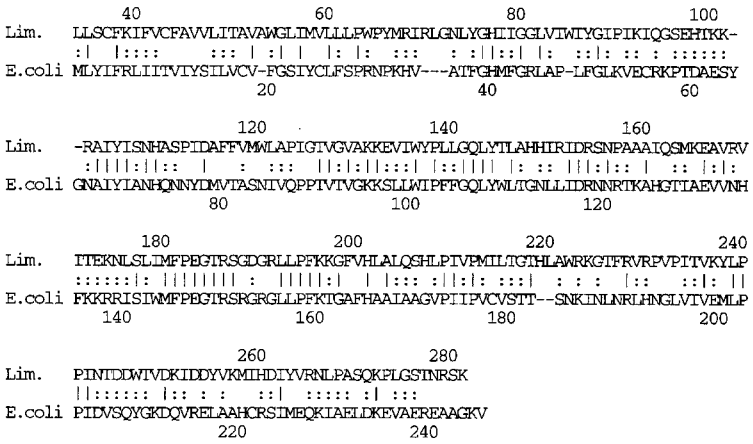

Fig. 3. Alignment of the deduced amino acid sequence of the largest ORF in pLAT2 with the *E. coli* LPA-AT protein-PlsC. Dashes show gaps introduced into the sequences to maximise identities. Vertical lines indicate exact matches between the two sequences and colons conservative amino acid changes. Lim. indicates the sequence of the ORF in pLAT2 and *E. coli* the sequence of PlsC. The numbers indicate amino acid residue positions in the full protein sequences.

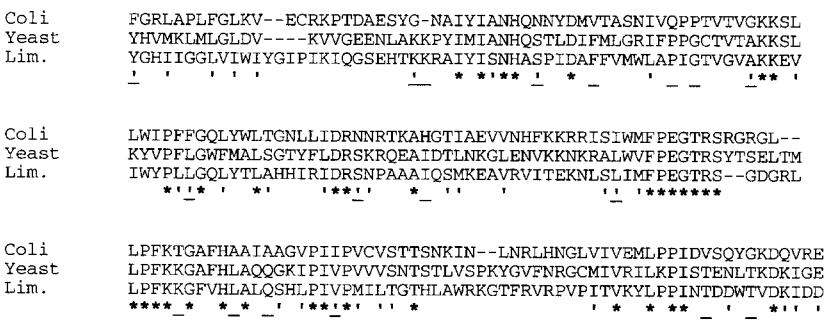

Fig. 4. Alignment of the *E. coli* and yeast putative LPA-AT sequences with the largest ORF in pLAT2 using CLUSTAL-V [12]. Dashes show gaps introduced into the sequences to maximise identities. Asterisks indicate identical and primes conserved amino acid residues in all three sequences; underlined residues show identities between SLC1 and the *L. douglasii* protein sequence. Lim. indicates the sequence of the ORF in pLAT2, yeast the sequence of SLC1 and *E. coli* the sequence of PlsC.

Northern and Southern analysis

A 900 bp *Eco* RI/*Sna* BI fragment of pLAT2, corresponding to all of the largest ORF in the cDNA was used as a probe against poly(A)$^+$ mRNA from seed, stem and leaf of *L. douglasii*. Transcription occurs predominantly in the developing embryo (Fig. 5) with a very low level of hybridisation apparent in leaf and stem only on very long exposure of the filter. The mRNA detected was 1.1 kb in size, indicating that the cDNA insert of pLAT2 is probably a full-length clone. When a duplicate northern blot was hybridised to the cDNA insert of pLAT1, hybridising bands 1.5 kb in size were observed in all three RNA samples (Fig. 5). Expression was highest in the stem, followed by leaf, with lower levels present in the embryo. Expression of this gene is not therefore localised to developing embryo material which is the case for the cDNA in pLAT2.

Southern blot analysis, using the whole *Eco* RI insert of pLAT2, was carried out with washing to a stringency of 80% similarity. The result (Fig. 6) indicated that the gene for the cDNA was a single copy gene, although limited hybridisation to another band did occur. The insert of pLAT1 on the other hand hybridised to several bands on an identically washed blot, indicating that the gene

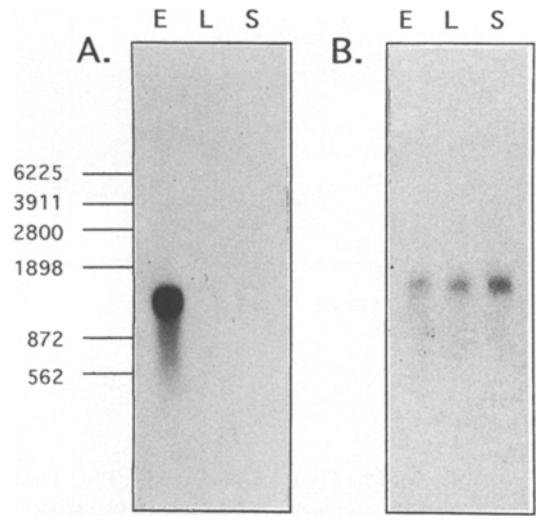

Fig. 5. Northern blot analysis of RNA from *Limnanthes*. 1 μg samples of poly(A)$^+$ RNA from embryo (lanes E), leaf (lanes L) and stem (lanes S) material of *L. douglasii* were separated and transferred onto Hybond N as described in the materials and methods. After hybridisation to DNA probes corresponding to the open reading frames of pLAT2 (panel A) and pLAT1 (panel B), the blots were washed in 0.1 × SSC, 0.1% SDS at 42 °C. Hybridisation was detected by autoradiography with Fuji RX film at −80 °C with an intensifying screen. Sizes of RNA markers are indicated.

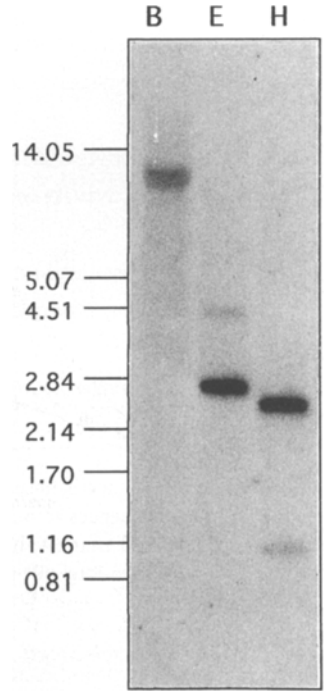

Fig. 6. Southern blot analysis of *Limnanthes* DNA. 5 μg samples of *L. douglasii* chromosomal DNA were digested with *Bam* HI (lane B), *Eco* RI (lane E) and *Hind* III (lane H). Fragments were separated by electrophoresis in a 0.7% agarose gel, transferred to Hybond N and then hybridised with the insert of pLAT2. The filter was washed in 2 × SSC, 0.1% SDS at 60 °C and autoradiography carried out as for Fig. 5.

for this cDNA was a member of a small gene family. This may be a reflection of the more widespread expression of this gene in the plant.

1-acyl-sn-*glycerol-3-phosphate acyltransferase assays*

Membranes were isolated from JC201(pLAT2) and JC200, which is isogenic to JC201 except it is *plsC*$^+$ [7]. Both cultures used for membrane preparation were grown at 30 °C in order to prevent any selective advantage to JC201(pLAT2) bacteria which could have reverted to *plsC*$^+$ during growth. The membranes were tested for their ability to incorporate either erucoyl-(22:1 Δ13) or oleoyl-(18:1 Δ9) CoA thioesters into phosphatidic acid using 1-erucoyl-*sn*-glycerol-3-phosphate as an acyl-acceptor. Membranes from JC200 had 7.4 times the activity of those isolated from JC201(pLAT2) when assayed for the ability to synthesize di-oleoyl phosphatidic acid. Both JC200 and JC201(pLAT2) membranes incorporate oleoyl CoA at a greater rate than erucoyl CoA, but it can clearly be seen (Fig. 7A) that those from JC201(pLAT2) are able to incorporate erucoyl-CoA more efficiently in relation to oleoyl-CoA than the membranes from JC200. The ratio of initial velocities for reactions with erucoyl-CoA and oleoyl-CoA were 1:1.32 for JC201(pLAT2) and 1:3.38 for JC200 membranes. In further experiments the ratios of initial velocities obtained were 1:1.38 and 1:1.45 for JC201(pLAT2) membranes and 1:3.3 and 1:3.44 for JC200 membranes. Addition of pLAT2 to JC201 therefore resulted in membranes having an entirely different specificity for erucoyl-CoA than membranes from *plsC*$^+$ bacteria. The restoration of 1-acyl-*sn*-glycerol-3-phosphate acyltransferase activity in JC201 used to prepare these mem-

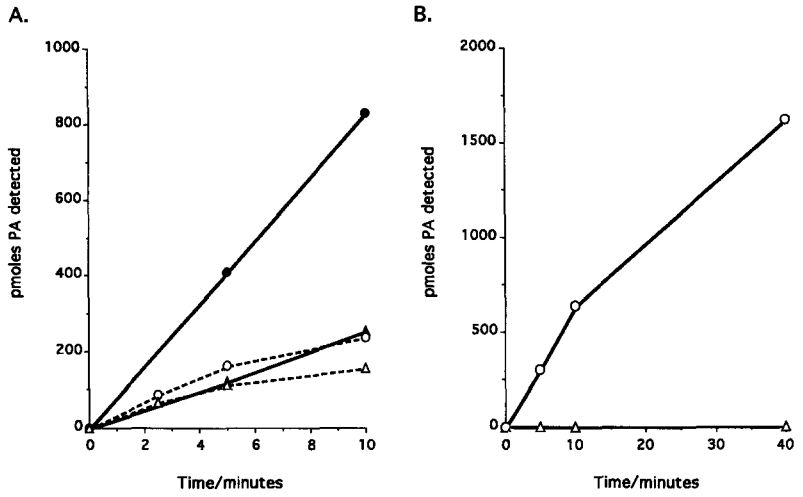

Fig. 7. 1-acyl-glycerol-3-phosphate acyltransferase activity of microsomes. The graphs show the amount of phosphatidic acid detected in 100 μl samples of assay mixtures as a function of the incubation time. A. Results obtained with membranes isolated from different *E. coli* strains. The *E. coli* strains and fatty acyl-CoA thioesters present in the incubation mixtures were as follows: ●—● JC200/18:1CoA; ▲—▲ JC200/22:1CoA; ○—○ JC201(pLAT2)/18:1CoA', △—△ JC201(pLAT2)/22:1CoA. B. Results obtained with rape microsomes. Acyl-CoA thioesters in the reaction mixture were: ○—○ 18:1 CoA', △—△ 22:1 CoA.

branes was not therefore due to reversion of bacteria to *plsC*$^+$, demonstrating that pLAT2 encodes a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase enzyme. Comparable experiments were carried out with high erucic acid rape microsomes using the same assay mixtures and 1-erucoyl-*sn*-glycerol-3-phosphate acyl acceptor. The microsomes demonstrated the ability to incorporate oleoyl-CoA at the *sn*-2 position of phosphatidic acid (Fig. 7B), but erucoyl-CoA was not incorporated to any significant level. The amount of phosphatidic acid detected after 40 minutes with erucoyl-CoA in the assay mixture was 0.43% of that detected when oleoyl-CoA was present.

Discussion

Complementation cloning and heterologous screening have resulted in the isolation of two different cDNA clones from developing seeds of *L. douglasii* which have similarities to LPA-AT sequences. The plasmid pLAT1 contains a cDNA which is a *Limnanthes* homologue of a maize sequence which complements JC201 [2]. pLAT1 is unable to complement the mutant itself however as the only large ORF present is preceded by a stop codon and no translation initiation signals are present to enable synthesis of any protein. Further studies on this plasmid were not undertaken.

Complementation of JC201 was used to select pLAT2, which encodes a protein, that by database searching, is most homologous to PlsC of *E. coli*. PlsC is the only protein which has been proved to be an LPA-AT enzyme on the basis of increased activity after protein over-expression [6]; although evidence strongly suggests that SLC1 of yeast, the second top matching protein, is the first eukaryotic LPA-AT sequence cloned [20]. The LPA-AT activity of membranes from complemented JC201 is lower than in those from *plsC*$^+$ bacteria even though the complementing protein is encoded on a high copy number plasmid. This could be due in part to inefficient translation initiation at the purine-rich region highlighted in Fig. 2 and codon usage differences or incorrect targeting of the protein to the bacterial membrane could also decrease the amount of activity detected.

The first 31 amino acids of the ORF in pLAT2, which are probably not translated in comple-

mented JC201, encode an extra hydrophilic domain not present in PlsC. This may act in the plant to target the protein to a specific location in the cell. This N-terminal region does have some features of secretory signal peptides [26] in that it contains a positively charged region (up to K-19, Fig. 2) and a central hydrophobic region (up to A-26, Fig. 2), although this is followed by charged rather than polar residues. This N-terminus may therefore be able to target the protein to the endoplasmic reticulum although there is no apparent cleavage site to suggest it is acting as a classical secretory signal peptide which is removed. The localisation of the protein encoded by the ORF in pLAT2 within the cell remains to be elucidated but it is unlikely that it is a chloroplastic protein as the N-terminus does not resemble a chloroplastic transit peptide [15, 26].

Nucleotide and amino acid sequence data showed that the cDNA inserts of pLAT1 and pLAT2 encode different proteins and northern blot analysis clearly demonstrate that the expression patterns in *L. douglasii* differ also. The cDNA in pLAT2 corresponds to a gene whose expression is seed-specific, indicating that its promoter is activated in a tissue-specific manner. The different expression patterns observed with pLAT1 and pLAT2 cDNAs may reflect the differing biological roles of the LPA-AT-like proteins they encode. That of pLAT1 may be an LPA-AT required for membrane phospholipid biosynthesis in all tissues, whereas that in pLAT2 may be necessary only for specific storage TAG biosynthesis in developing seed.

The specificities for oleoyl-CoA and erucoyl-CoA of membranes from complemented JC201 have been determined with a microsomal assay system. Since we were interested in identifying an LPA-AT from *Limnanthes* that was capable of making dierucoyl phosphatidic acid, 1-erucoyl-*sn*-glycerol-3-phosphate was used as an acyl-acceptor. This was labelled with ^{32}P to simplify analysis of the phosphatidic acid products. LPA-AT assays described in the literature have used a number of different conditions and the method described here is a modification of the assay for LPA-AT in *E. coli* membranes [7]. These membranes contain the phosphatidyl glycerophosphate B phosphatase (PgpB) which is a non-specific phosphatase capable of hydrolyzing phosphatidyl glycerophosphate, lysophosphatidic acid (LPA) and phosphatidic acid (PA) [13]. PgpB did not appear to be active in the assay system used however, as the amount of ^{32}P-phosphate present in the assay mixtures, measured in both the aqueous layer after lipid extraction and at the origin after development of TLC plates, did not increase with either increasing incubation time or amount of membranes added. No variation in the low levels of ^{32}P-phosphate present was apparent when oleoyl-CoA or erucoyl-CoA were used in assays and any differences in amount of PA detected was not therefore due to variable activity of PgpB on 1-erucoyl-*sn*-2-oleoyl PA and dierucoyl PA. The amount of membrane fractions added to the assays was such that less than 10% of the LPA and CoA were converted to PA after ten minutes. Initial velocities could be determined from the results obtained but it is unclear why linear rates of incorporation do not continue with membranes from JC201(pLAT2) bacteria after 5 minutes (Fig. 7A). This may be due to the micellar nature of both substrates and the product or the K_m value for the enzyme, which is unknown. It was not due to the use of different substrate preparations, which may produce different results with micellar substrates, as the identical 1-erucoyl-*sn*-glycerol-3-phosphate preparation was used in assays for membranes from both *E. coli* sources. The results clearly show that the complemented JC201 contain an LPA-AT with a different specificity for erucoyl-CoA than membranes from *plsC*$^+$ *E. coli*. The observed ratios of amount of PA produced with oleoyl- and erucoyl-CoA thioesters are similar to reported data obtained with microsomes from seeds of *L. douglasii*. where the ratio of acylation rates with erucoyl-CoA and oleoyl-CoA was 1:1.2–1.3 over a range of CoA concentrations [18]. The results of sequence alignment strongly indicate that pLAT2 encodes an LPA-AT enzyme, which is confirmed by the different specificities seen in the acyltransferase assays.

Rape microsomes were also included in the

experiments as a control for the methods developed. The data obtained confirmed previous results [1] and showed that the LPA-AT in high erucic acid rape strains discriminates against incorporation of erucoyl-CoA. The activity of PlsC with different CoA species has, to our knowledge, not been reported and the results of these experiments interestingly show that this enzyme is able to synthesize dierucoyl PA, although the rate of incorporation of oleoyl-CoA is much greater than erucoyl-CoA. By far the preferred candidate gene for transfer into rape to produce trierucin is the insert of pLAT2, which encodes an enzyme with little discrimination between erucoyl-CoA and oleoyl-CoA. It is likely that there are higher levels of erucoyl-CoA than oleoyl-CoA in high erucic acid rape cultivars, since the glycerol-3-phosphate- and diacylglycerol-acyltransferases show little discrimination between CoA species and high levels of erucic acid are present at the *sn* 1 and 3 positions of their TAGs. Transfer of the LPA-AT gene of pLAT2 into such rape varieties is currently in progress and may produce plants capable of synthesizing the desired product trierucin in their seed oils.

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