Cloning and Characterization of a Constitutive *Lysophosphatidic Acid Acyltransferase 2* (*LPAT2*) Gene from *Tropaeolum majus* L

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Abstract: The cloning and characterization of a *lyso*-phosphatidic acid acyltransferase (LPAT2; EC 2.3.1.51) from *Tropaeolum majus* from a 20,000 EST collection is described. The 1358 bp *TmLPAT2* gene encodes a 42.6 kD polypeptide; the primary sequence has a membrane bound *O*-acyltransferase, MBOAT, Box I motif, and Boxes II, III and IV typical of LPATs from various species. Unlike many *LPAT2*s, the gene was constitutively expressed in all tissues. The TmLPAT2 functionality was confirmed by expression in a yeast LPAT deletion (*SLC1*¯) mutant. The TmLPAT2 could use a range of acyl-CoAs as acyl donor, including 22:1-CoA and 20:1-CoA and either 18:1-LPA or 22:1-LPA as acyl acceptor. This new *LPAT2* could enable the production of *Brassica* seed oils with enhanced levels of very long-chain fatty acids.

Keywords: *Tropaeolum majus*, LPAT, *lyso*-phosphatidic acid acyltransferase, erucic acid, triacylglycerol, yeast.

INTRODUCTION

Many groups worldwide have a vested interest in inserting genes into *Brassica napus* in an effort to produce the industrial feedstock trierucin. Erucic acid (*cis*-13 docosenoic acid, 22:1) is the major very long-chain fatty acid (VLCFA) in the seed oil from HEAR (high erucic acid rapeseed) *B. napus* cultivars, accounting for 45-55% of the total fatty acids [1]. HEAR cultivars are of high interest for industrial purposes because 22:1 is a valuable feedstock with more than 1000 potential or patented industrial applications [2, 3]. Currently the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an anti-block or slip promoting agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid, e.g. in lubricants, detergents, film processing agents and coatings, as well as in cosmetics and pharmaceuticals [4-7]. For many of these industrial uses, the economics are limited by the proportion of 22:1 in the seed oil, approximately 45%. To compete with petroleum-based products, it is desirable to increase the 22:1 proportion as high as possible in order to reduce the cost of purification [3]. In addition, the engineering of HEAR *Brassicaceae* to produce seed oils containing substantial trierucin would lend the intact oil to a wide range of new applications [2]. In general, stereospecific analyses have shown that among most members of the *Brassicaceae*, 22:l is virtually excluded from the *sn*-2 position of TAGs [8]; thus erucic acid is essentially found only in the *sn*-3 and the *sn*-1 positions, limiting the overall proportions of 22:1 to a maximum of about 66 mol %. The best HEAR *B. napus* spring cultivars have only about 55% erucic acid in the seed oil, but again, the proportion in the *sn*-2 position is negligible [7].

In the traditional Kennedy pathway for seed oil (triacylglycerol, TAG) biosynthesis, *lyso*-phosphatidic acid acyltransferase (LPAT; EC 2.3.1.51) is the major enzyme responsible for acylating the *sn*-2 position of the glycerol backbone and therefore largely determines the *sn*-2 acyl composition of TAGs [9, 10]. This presumably ER-based LPAT is typically referred to as an LPAT2 in most oilseed species, distinguishing it from the plastidial LPAT1 which has enzyme characteristics more like the prokaryotic LPAT found in *E. coli* (See overview by Kim *et al.*, [11]).

Several studies have suggested that in *B. napus* the limitation with respect to erucic acid proportions at the *sn*-2 position is at least partially due to the inability of the LPAT2 to utilize erucoyl-CoA [12-15]. Various groups worldwide have attempted or advocated the transformation of rapeseed with an LPAT2 gene which has the desired capacity to utilize erucoyl-CoA during TAG bioassembly [14-19]. LPAT2s from *Limnanthes* spp appear to be capable of inserting erucoyl moieties into the *sn*-2 position. While the overall proportions of erucic acid in the seed oil of *Limnanthes* is only about 16%, of this, about 60% is found in the *sn*-2 position [14-19]. Accordingly, LPAT2s from *L. douglasii* and *L. alba* have been cloned and used to enhance *sn*-2 erucic proportions in *B napus* [20-22] but with this single genetic modification, the enhancement of overall proportions of erucic acid and accumulation of significant trierucin have not resulted [23]. Indeed, the erucic acid was merely redistributed from the *sn*-1 and *sn*-3 positions to the *sn*-2 position, with no significant improvement in the mol % erucic acid in seed TAGs. This suggested, that in part, erucic acid supply was a limiting factor. Recently it has been shown that by combin-

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ing alleles of *B. napus* related to low polyunsaturated oils with the transgenic co-expression of the *L. douglasii LPAT2* and over-expression of the *BnFAE1* (encoding 3-ketoacyl-CoA synthase, catalyzing the first condensation reaction in the elongase complex which synthesizes erucic acid), a *B. napus* line with oil containing just over 70% erucic acid was obtained, clearly a breakthrough [24].

The only species so far identified that naturally possesses erucic acid proportions of 75% or greater, mainly as trierucin, in its seed oil is garden nasturtium (*Tropaeolum majus* L.). Nasturtium is not an oilseed *per se*, accumulating only about 10-20% oil, but of this, 80-85% is trierucin. Thus, we chose *T. majus* as a model system for studying trierucin bioassembly so that this capacity could be engineered in a *Brassica* oilseed. It was in this context that we undertook the task of creating an EST collection of *T. majus* sequences in an effort to isolate and define specific genes which can contribute to trierucin synthesis. These nasturtium genes may provide a means to engineer rapeseed and other *Brassica* oilseeds to produce ultra-high erucic acid (80% or greater) oils. In our analysis of our 20,000-EST collection, we found two overlapping contigs which, when re-assembled, gave us a clone encoding a putative *T. majus LPAT2* (*TmLPAT2*). We describe herein, its isolation and characterization by expression in a yeast *LPAT*$^{-}$ (*slc*Δ) deletion strain, and the implications for its utility in engineering new oilseed profiles.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tropaeolum majus seeds (cultivar Dwarf Cherry Rose) were obtained from Early's Farm and Garden Centre, Saskatoon, SK, and were grown at the Kristjanson Biotechnology Complex greenhouses, Saskatoon, under natural light conditions supplemented with high-pressure sodium lamps with a 16 h photoperiod (16 h of light and 8 h of darkness) at 22ºC and a relative humidity of 25 to 30%. Flowers were hand-pollinated and seeds at various stages of development were harvested, their seed coats were removed and embryos were frozen in liquid nitrogen and stored at -80°C. The lipid composition of developing nasturtium embryos at various stages of development was determined. The following were the designated stages of embryo development in days post anthesis: Early: 8-12 d.p.a.; Early-mid: 13-20 d.p.a.; Mid: 22-27 d.p.a.; Mid-late: 27-30 d.p.a.; Mature: 35 d.p.a. Arabidopsis thaliana plants were grown and harvested as described previously [25].

Analysis of Oil Accumulation in Developing *Tropaeolum majus* Embryos

Freeze-dried *T. majus* embryos of early-, mid- and late-developmental stages, as well as mature seeds, were weighed and transferred to a cooled mortar and ground in 2 ml CH_2Cl_2:IPA (2:1). The mixture was transferred to a test tube to which was added the above solvent (1ml) and 0.9% NaCl (1ml) before vortexing; 2 ml CH_2Cl_2 was added, the mixture re-vortexed and centrifuged at 2500 r.p.m. for 3 min. The CH_2Cl_2 layer was removed, the extraction repeated and the CH_2Cl_2 layers combined. $CHCl_3$: Benzene: Methanol (1:1:1) (1ml) was added and then the sample evaporated to dryness. The dried sample was resuspended in $CHCl_3$ (1 ml) to give the total lipid extract (TLE). The developmental acyl composition of the TLE and the total oil content at each stage were determined by transesterification followed by GC using tri-15:0 as an internal standard and tri-17:0 as an external standard (to determine completeness of transmethylation) as described previously [25]. The *T. majus* TAG fraction was isolated by TLC and a stereospecific analysis performed as described by Taylor *et al*., [26].

T. majus cDNA Library Construction and Normalization

cDNA was synthesized from mRNA isolated from mid-developing nasturtium embryos using a cDNA synthesis kit (Stratagene). The cDNA was directionally cloned into the pBluescript SK II (+) vector (Stratagene) and transformed into DH10B electrocompetent cells. The primary library was amplified using semi-solid agar (SeaPrep agarose, Mandel). Normalization of the library was performed at C_0t 2.5 and C_0t 5 following the normalization method 4 of Bonaldo *et al*., [27]. Double-stranded phagemid DNA was converted to single stranded DNA using Gene II protein and Exonuclease III (Genetrapper cDNA Positive Selection System, Gibco BRL, cat. no. 10356-020). The single stranded DNA was purified from the double stranded DNA using HAP chromatography (type II Hydroxyapatite, BioRad, cat. no. 158-4200). 20,000 ESTs from this library were sequenced as described below.

Sequencing and Analysis

Sequencing was performed on an ABI3730xl DNA Analyzer using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequence analyses were performed using Lasergene software (DNAStar, Madison, WI, USA). Sequence similarity searches and other analyses were performed using BLASTN, BLASTX [28] and PSORT [29] programs. The *T. majus LPAT2* clone was represented by 2 overlapping contig members among 20,000 ESTs isolated and analyzed from a normalized cDNA library prepared from mid-developing nasturtium embryos.

Cloning of Full Length *T majus* (*Tm*) and *A thaliana* (*At*) *LPAT2* cDNAs and Assembly of Yeast Transformation Constructs

TmLPAT2

Primers were designed to amplify the *TmLPAT2* gene with adaptor restrictions sites (in italics): 5' primer amplifying *TmLPAT2* sequence with a Kpn I restriction site (GA*GGTACC*GGAAATGTCAGTTGCAGC); 3' primer amplifying *TmLPAT2* sequence (+ stop codon) with an Xho I site (CCG*CTCGAG*TTTTACTGATGTTTGGTTGC). cDNA was synthesized from total RNA extracted from *T. majus* mid-developing embryos using Superscript II (Invitrogen). The *TmLPAT2* gene was amplified from the cDNA using Turbo Pfu polymerase (Stratagene) on a RoboCycler thermocycler (Stratagene) using the following program: 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 2 min, an additional extension of 72°C for 10 min was included. The amplified PCR product was digested using restriction enzymes KpnI (NEB) and XhoI (NEB). The PCR-amplified *TmLPAT2* was directionally cloned into the

respective KpnI and XhoI sites of pYES/NT B (Invitrogen) using T4 DNA ligase (Invitrogen) in a 4:1 molar ratio (insert: vector) at 4°C overnight. Four microliters of the ligation reaction were used to transform Top10 Chemically Competent Cells (Invitrogen) as per standard protocols. Putative clones were cultured in liquid medium with ampicillin selection, and grown overnight at 37°C with shaking (250 rpm). Plasmid DNA was extracted using a QIAprep Spin miniprep kit (Qiagen). PCR screening for the gene insert was performed using GAL1/V5CR primers and Taq polymerase (Program: 95° - 3 min; 30 cycles of 95°C – 30s, 52°C -45s, 72°C-2 min; 72°C-10 min). Positive clones were confirmed by sequencing.

At LPAT2

Primers were designed to amplify the *A. thal LPAT2* gene (Accession No. At3g57650) with adaptor restrictions sites (in italics): 5' primer amplifying *AtLPAT2* sequence with a Kpn I restriction site (CC*GGTACC*AGGATGGTGATTGC-TGCAGCT); 3' primer amplifying *AtLPAT2* sequence (+ stop codon) with an Xho I site CC*CTCGAG*TGTGAGAAC-CAGTTTTTACTT). Total RNA was extracted from *A. thaliana* ecotype Columbia leaves using Trizol (Invitrogen) and the cDNA was synthesized and used to amplify the *A thaliana LPAT2* as described for the *TmLPAT2* above.

Molecular Analyses of the *TmLPAT2*

The alignment of the *TmLPAT2* with other cloned *LPAT*s shown in Fig. (**2**) and the phylogenetic tree with bootstrap values (phenogram) shown in Fig. (**3**), were calculated using the Clustal W method in the Lasergene Megalign analysis software suite (DNAStar, Madison, WI.).

For Southern analysis, total DNA was extracted from 1 g of *T. majus* leaves according to Dellaporta *et al*., [30]. About 15 ug of genomic DNA were digested with restriction enzymes AseI, EcoRI, HindIII and XhoI (NEB) and the fragments separated by agarose gel (0.7%) electrophoresis in 1X TAE buffer. The DNA was blotted onto Amersham Hybond XL (GE Healthcare) membrane using a Turbo Blotter (Whatman) and following the alkaline transfer procedure (0.4 N NaOH). The Southern probe was amplified by PCR with primers designed to bind to a 450 bp region from the 5′end of the *KCS* gene and that would not be cut by the restriction enzymes selected here. Hybridization was carried out in Denhardt's buffer at 65 ^{0}C for 16 h using the ^{32}P-labeled DNA probe using a Random Primers labeling kit (Invitrogen). High stringency washings were performed at 65 ^{o}C (2X 15 min with 2X SSC/0.1 % SDS and 1X 15 min with 0.1 X SSC/0.1 % SDS) before exposing to X-ray film (Fuji Medical X-Ray film) at -80 ^{0}C.

In studies of *TmLPAT2* expression by northern analysis, total RNA was extracted using either Trizol Reagent (Invitrogen) for root, stem and leaf tissues, or using the method of Wang and Vodkin [31] for flower and embryonic (three developmental stages) tissues. 20 µg of each sample were loaded onto a 1.2 % denaturing formaldehyde agarose gel. The RNA was transferred onto a Nytran SuPerCharge membrane in 10 X SSC using a Turbo Blotter (Whatman) and hybridized as above using a full length *TmLPAT* cDNA as a probe. High stringency washings were performed at 60 ^{0}C, 2X 15 min with 40 mM NaPO4/1 mM Na EDTA/0.5 % SDS and 2X 15 min with 40 mM NaPO4/1 mM Na EDTA/0.1 % SDS before exposing to X-rays at -80 ^{0}C.

Transformation of Y03749 Yeast *LPAT*⁻ Deletion Mutant Strain with T*mLPAT2* or *AtLPAT2*

Yeast strain Y03749 (*slc1Δ, MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDL052c::KanMX4)* was transformed with the pYES2 NT B-*AtLPAT2* or with pYES2 NT B-*TmLPAT2* or with empty pYES2 NT B vector using the small scale yeast transformation method from the pYES2 NT B manual. Clones were selected on plates contaning (SC –Uracil + 2% Glucose).

Transformation was confirmed as follows: DNA from putative positive clones was extracted using a quick plasmid extraction method. A single colony was picked into 200 µl lysis buffer (100mM NaCl, 10mM Tris-HCL pH8.0, 1mM EDTA, and 0.1% SDS). An equal volume of acid-washed beads (0.5mm diameter) was added along with 200 µl phenol:choloroform (2:1), the sample vortexed for 30 seconds, followed by slow rotation for 5 minutes to complete the extraction. The mixture was then centrifuged for 5 minutes at 13000 rpm. The aqueous phase was recovered and the DNA precipitated using 2 volumes of ethanol and 0.1 x volume of 3M sodium acetate. The sample was incubated at -80°C for 30 minutes then centrifuged at 13000 rpm for 15 min. The DNA pellet was washed with 80% ethanol, re-centrifuged at 13,000 rpm for 5 min, the DNA pellet air dried and resuspended in 50 µl TE buffer. DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer. Top10 Chemically Competent cells (Invitrogen) were transformed with the purified DNA as per a standard protocol. Colonies were screened by PCR and restriction digests.

TmLPAT2 and *AtLPAT2* Expression in Y03749 Yeast *LPAT*⁻ *(slcΔ)* Deletion Mutant and Assay of LPAT Activity

Starter cultures of the Y03749 *TmLPAT2*, Y03749 *AtLPAT2* or Y03749 plasmid only (control) transformants were grown in minimal medium (SC –Uracil + 2% Glucose) at 30°C with shaking at 250 rpm. Culture density was measured using OD_{595}, and sufficient culture to produce an OD_{595} =0.4 in 50ml of Induction Medium (SC –Uracil + 2% Galactose + 1% Raffinose) was centrifuged, washed and resuspended in Induction Medium. Cultures were induced at 30°C with shaking (250 rpm) for 8, 16, 24 or 48 hrs. The maximum expression of the *Tm*LPAT2 recombinant protein was obtained after a 24 hour induction; the *At*LPAT2 expression was maximal at 48 hr of induction. Induced cells were harvested by centrifugation and protein was extracted using the glass bead/bead beater method. Protein concentration was measured in triplicate using Bradford reagent, normalized and the protein was used immediately for the LPAT assay.

LPAT assays of the recombinant protein fraction from each transformant were conducted as described previously [26] using either ^{14}C 18:1-CoA or ^{14}C 22:1-CoA as the acyl donor and 18:1-LPA or 22:1-LPA as the acyl acceptor. The resolution of the ^{14}C-labeled PA product by TLC run in ethyl acetate/iso-octane/acetic acid (45/15/10) was as described by Taylor *et al.* [26].

motifs and secondary or tertiary structure which may define acyl donor and acceptor preferences.

CONCLUSIONS

In summary, our findings would suggest that *Tm*LPAT2 does indeed possess a strong capacity to utilize 22:l-CoA. Combined with other studies of the biosynthesis of TAGs containing *sn*-2 22:l in *T. majus* [40-42], it appears that such TAGs are probably synthesized *via* the Kennedy pathway and not *via* acyl exchange at the *sn-2* position of DAGs and TAGs as suggested previously [39]. Thus, in all oilseeds studied thus far that accumulate TAGs containing VLCMFAs, the data are consistent with VLCMFA biosynthesis by elongation of oleoyl moieties to give VLCMFA-CoAs, which are then used in the synthesis of TAGs *via* the Kennedy pathway acyltransferases.

We propose that the capacity to acylate the glycerol backbone with erucoyl moieties at the *sn*-2 position, is a very valuable property for molecular breeding of high erucic acid plants. For example, the expression of the *TmLPAT2* clone in high erucic *Brassicaceae* (e.g. the high erucic *B. carinata* lines co-expressing the *Crambe KCS* and an RNAi-silenced *B. carinata FAD 2* [43] or the *Cardamine graeca KCS* [45], will be capable of enhancing the overall proportions of VLCFAs in the *sn*-2 position of TAGs and increase the probablility of producing trierucin or trinervonin, respectively. In addition, the transgenic over-expression of the *TmLPAT2* in the *Brassicaceae* may afford improvements in oil content in a manner not dissimilar to that already shown for ectopic expression of the yeast *SLC1-1* gene [44]). Accordingly, transformation experiments are currently underway in our lab to test these hypotheses.

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ABBREVIATIONS

A thal	=	(Arabidopsis thaliana)
CoA	=	Co-enzyme A
cDNA	=	Complementary DNA
EST	=	Expressed sequence tag
LPA	=	*Lyso*-phosphastidic acid
LPCAT	=	*Lyso*-phosphatidylcholine acytransferase
LPAT 2	=	*Lyso*-phosphastidic acid acyltransferase 2 (E.C. EC 2.3.1.51), *sn*-2 acyltransferase
MBOAT	=	Membrane bound *O*-acyltransferase
PA	=	Phosphastidic acid
SLC1-1$^-$	=	Yeast *lyso*-phosphastidic acid acyltransferase deletion mutant, slcΔ
T. majus	=	*Tropaeolum majus* (garden nasturtium)
TAG	=	Triacylglycerol
VLCFA	=	Very long-chain fatty acid
22:1 Erucic acid (22:1 c13; 22:1 Δ13)	=	all fatty acids are similarly designated x:y, carbon chain length: number of double bonds

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