THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 29, pp. 18068–18078, July 17, 2015


# *In Vivo* and *in Vitro* Evidence for Biochemical Coupling of Reactions Catalyzed by Lysophosphatidylcholine Acyltransferase and Diacylglycerol Acyltransferase*

Received for publication, March 27, 2015, and in revised form, June 5, 2015 Published, JBC Papers in Press, June 8, 2015, DOI 10.1074/jbc.M115.654798

**Xue Pan**[‡1], **Guanqun Chen**[‡], **Michael Kazachkov**[§], **Michael S. Greer**[‡], **Kristian Mark P. Caldo**[‡], **Jitao Zou**[§], **and Randall J. Weselake**[‡2]

*From the* [‡]*Alberta Innovates Phytola Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2P5, Canada and* [§]*National Research Council Canada, Saskatoon, Saskatchewan S7N 0W9, Canada*

**Background:** Plant polyunsaturated fatty acids (PUFAs) are mainly synthesized on phosphatidylcholine (PC).
**Results:** Diacylglycerol acyltransferase (DGAT) produced higher amount of PUFA-containing TAG in the presence of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT).
**Conclusion:** The LPCAT-catalyzed reverse reaction can be coupled to the DGAT reaction for PUFA accumulation.
**Significance:** A mechanism for enhancing the transfer of PUFAs from PC into TAG has been confirmed.

**Seed oils of flax (*Linum usitatissimum* L.) and many other plant species contain substantial amounts of polyunsaturated fatty acids (PUFAs). Phosphatidylcholine (PC) is the major site for PUFA synthesis. The exact mechanisms of how these PUFAs are channeled from PC into triacylglycerol (TAG) needs to be further explored. By using *in vivo* and *in vitro* approaches, we demonstrated that the PC deacylation reaction catalyzed by the reverse action of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) can transfer PUFAs on PC directly into the acyl-CoA pool, making these PUFAs available for the diacylglycerol acyltransferase (DGAT)-catalyzed reaction for TAG production. Two types of yeast mutants were generated for *in vivo* and *in vitro* experiments, respectively. Both mutants provide a null background with no endogenous TAG forming capacity and an extremely low LPCAT activity. *In vivo* experiments showed that co-expressing flax *DGAT1-1* and *LPCAT1* in the yeast quintuple mutant significantly increased 18-carbon PUFAs in TAG with a concomitant decrease of 18-carbon PUFAs in phospholipid. We further showed that after incubation of *sn*-2-[$^{14}$C]acyl-PC, formation of [$^{14}$C]TAG was only possible with yeast microsomes containing both LPCAT1 and DGAT1-1. Moreover, the specific activity of overall LPCAT1 and DGAT1-1 coupling process exhibited a preference for transferring $^{14}$C-labeled linoleoyl or linolenoyl than oleoyl moieties from the *sn*-2 position of PC to TAG. Together, our data support the hypothesis of biochemical coupling of the LPCAT1-catalyzed reverse reaction with the DGAT1-1-catalyzed reaction for incorporating PUFAs into TAG. This process represents a potential route for enriching TAG in PUFA content during seed development in flax.**

Seed oils are major plant-based sources of essential polyunsaturated fatty acids (PUFAs)[3] for human diet and many industrial applications. Understanding the fundamental aspects of how plant fatty acids are synthesized in seed oils is essential for developing new strategies to alter fatty acid composition for improved nutritional quality and industrial uses of seed oils.

In oilseeds, fatty acid synthesis occurs exclusively in the plastids and newly synthesized fatty acids, mostly as oleic acid (18:1$^{cis\Delta9}$), palmitic acid (16:0), and stearic acid (18:0), and are then almost entirely (>95%) exported to the cytoplasm to enter into the acyl-CoA pool (1). In the cytoplasm, the 18:1 from the acyl-coenzyme A (CoA) pool is quickly esterified to the phosphatidylcholine (PC) of the endoplasmic reticulum. Desaturation of 18:1 to linoleic acid (LA; 18:2$^{cis\Delta9,12}$) and then α-linolenic acid (ALA; 18:3$^{cis\Delta9,12,15}$) occurs through the catalytic action of endoplasmic reticulum-localized fatty acid desaturases, the oleate desaturase (FAD2), and the linoleate desaturase (FAD3), producing PUFA-enriched PC (2). It has been shown that PC also is the major site that synthesizes a number of unusual fatty acids, such as hydroxy and conjugated fatty acids (3).

As shown in Fig. 1, various pathways have been proposed for transferring PUFAs from PC into triacylglycerol (TAG), which is the main component of seed oil. PUFAs may be directly transferred from PC into TAG via the catalytic action of phospholipid:diacylglycerol acyltransferase (PDAT) (4). Some oilseeds that are high in PUFAs like flax (5) or unusual fatty acids like castor (*Ricinus communis*) (6, 7) appear to have unique PDAT(s) with high selectivity toward these fatty acids. Alternatively, the phosphocholine head group of PUFA-enriched PC may be removed, producing PUFA-enriched diacylglycerol (DAG), which may be further incorporated into TAG via either

* The authors declare that they have no conflicts of interest with the contents of this article.

[1] Recipient of an Alberta Innovates Doctoral Award.

[2] Supported by Genome Alberta/Genome Canada, the Canada Research Chairs Program, the Canada Foundation for Innovation, the Research Capacity Program of Alberta Enterprise and Advanced Education, AVAC Ltd., Alberta Innovates Bio Solutions, and the Natural Sciences and Engineering Research Council of Canada. To whom correspondence should be addressed. Tel.: 780-492-4401; E-mail: randall.weselake@ualberta.ca.

[3] The abbreviations used are: PUFA, polyunsaturated fatty acid; DGAT, acyl CoA:diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; TAG, triacylglycerol; FAD, fatty acid desaturase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; PLA, phospholipase A; DAG, diacylglycerol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LA, linoleic acid; ALA, α-linolenic acid; $K_s$, synonymous substitution rate; Lyso-PAF, lyso-platelet-activating factor; SC-Ura, synthetic complete medium lacking uracil.

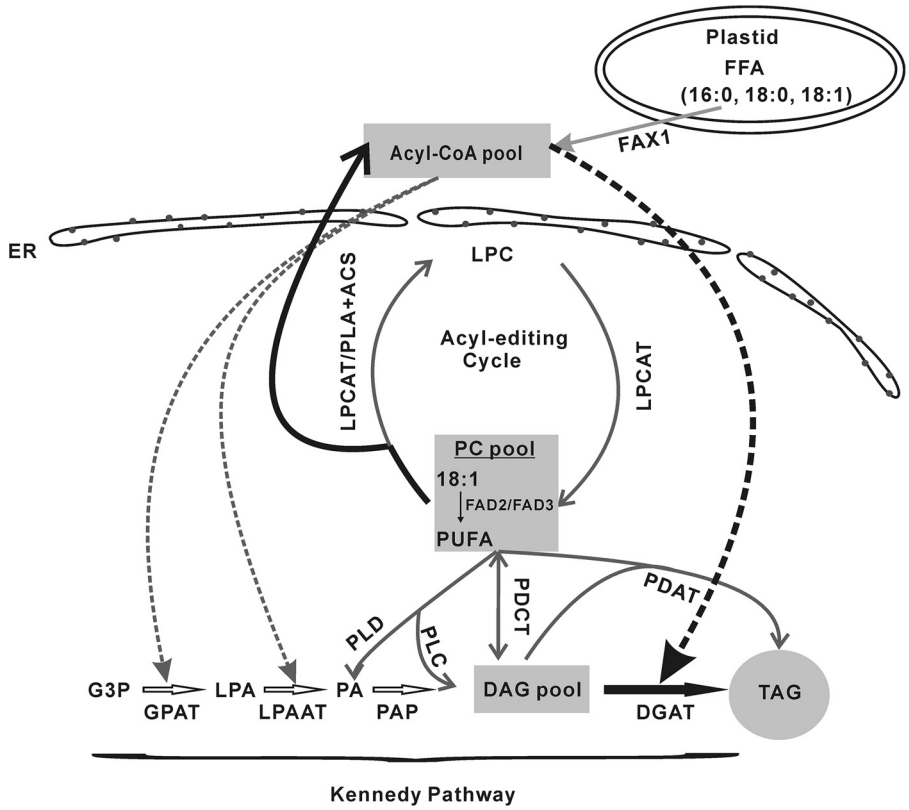



FIGURE 1. **A schematic diagram showing the proposed mechanisms involved in transferring PUFAs from PC into TAG.** The Kennedy pathway for TAG synthesis is indicated by *white arrows*, and acyl transfer reactions are shown in *dashed lines*. The transfer of PUFAs from PC into TAG catalyzed by coupled reactions of the LPCAT-catalyzed reverse reaction and the DGAT-catalyzed reaction is highlighted in *black bold*. *ACS*, acyl-CoA synthetase; *ER*, endoplasmic reticulum; *FAX1*, fatty acid export 1; *FFA*, free fatty acid; *G3P*, glycerol-3-phosphate; *GPAT*, acyl-CoA:glycerol-3-phosphate acyltransferase; *LPA*, lysophosphatidic acid; *LPAAT*, acyl-CoA:lysophosphatidic acid acyltransferase; *PAP*, phosphatidic acid phosphatase; *PDCT*, phosphatidylcholine:diacylglycerol cholinephosphotransferase; *PLC*, phospholipase C; *PLD*, phospholipase D.

or both of PDAT and diacylglycerol acyltransferase (DGAT)-catalyzed reactions. The conversion of PUFA-rich PC to PUFA-rich DAG may also involve the catalytic action of phospholipase C, phospholipase D along with phosphatidic acid phosphatase, and the recently discovered phosphatidylcholine: diacylglycerol cholinephosphotransferase (8). In addition, PUFAs may be removed from PC and enter the acyl-CoA pool via acyl-exchange predominately between the acyl groups at the *sn*-2 position of PC and the acyl-CoA pool mediated by the combined forward and reverse reactions catalyzed by acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) (9). PUFA-enriched acyl-CoA may also be generated through liberation of free fatty acid from the *sn*-2 position of PC by phospholipase A (PLA) followed by activation of free fatty acid to acyl-CoA through acyl-CoA synthetase with LPCAT acting in the forward reaction to catalyze the re-acylation of lysophosphatidylcholine (LPC) to PC in a process known as the "Lands cycle" (10, 11). The resulting PUFA-CoAs may be used by enzymes involved in the Kennedy pathway to form PUFA-enriched TAG. Recently, by using reverse genetics and metabolic labeling approaches, it was demonstrated that LPCAT-mediated acyl-editing together with phosphatidylcholine:diacylglycerol cholinephosphotransferase-based PC-*sn*-1,2-diacylglycerol interconversion control the major fluxes of PUFAs from PC to TAG (~66%) in *Arabidopsis* seeds (12). In addition, *in vitro* evidence has shown that plant LPCAT does indeed operate in a reversible fashion, even though the reverse reaction is a thermodynamically unfavorable process (13). Despite recent progress, direct evidence of how LPCAT interacts with the enzymes involved in the Kennedy pathway is still lacking.

In this study we focused on the interaction between flax LPCAT1 and a type 1 DGAT. LPCAT can catalyze both forward and reverse reactions (*Reaction 1*, Fig. 2). The reverse reaction of LPCAT uses PC as substrate and forms acyl-CoA and LPC. DGAT catalyzes the final step in the Kennedy pathway of TAG synthesis from the substrates acyl-CoA and DAG (*Reaction 2*, Fig. 2). The reverse reaction of LPCAT and the DGAT reaction share a common intermediate, acyl-CoA.

Based on Reactions 1 and 2 (Fig. 2), we proposed that the PUFA synthesized on PC can be removed and transferred to the acyl-CoA pool by the reverse reaction of LPCAT and then be further utilized by the DGAT-catalyzed reaction for TAG biosynthesis. As highlighted in Fig. 1, the overall reaction represents a route for transferring PUFAs from PC to the acyl-CoA pool and then to TAG. We chose to test this hypothesis through heterologous expression of flax genes in yeast mutant background devoid of LPCAT activity and TAG-forming capacity. Flax oil naturally contains substantial amount of PUFAs, especially ALA. To accumulate a large proportion of PUFAs in seed oils, flax may have more efficient mechanisms to channel PUFAs from PC into TAG as compared with other plant species. Many TAG synthesis-related genes from flax are closely

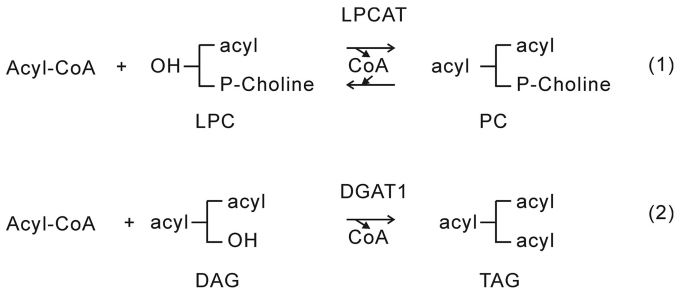



FIGURE 2. **A schematic diagram showing the LPCAT-catalyzed forward and reverse reactions (*Reaction 1*) and the DGAT-catalyzed reaction for TAG synthesis (*Reaction 2*).**

related to genes found in plant species that produce high amounts of unusual fatty acids, such as castor and tung tree (*Vernicia fordii*) (14). The PUFA-enriching mechanism found in flax may also represent a route for enriching unusual fatty acids in these species.

Taken together with earlier reported *in vitro* data on plant LPCAT action (13), our data further confirm that plant LPCAT can act in a reversible fashion under *in vivo* condition. These data demonstrate for the first time *in vivo* evidence of the reversibility of the LPCAT. More interestingly, our *in vivo* and *in vitro* data support the hypothesis of biochemical coupling of the reverse reaction catalyzed by LPCAT with the DGAT-catalyzed reaction for the incorporation of PUFAs into TAG. These results contribute to our understanding of the mechanisms responsible for channeling PUFAs from PC into TAG in oleaginous plants that produce PUFA-enriched TAG during seed development.

## Experimental Procedures

*Chemicals*—[1-$^{14}$C]18:0, [1-$^{14}$C]18:1, [1-$^{14}$C]18:2, [1-$^{14}$C]18:3, and [$^{14}$C]18:1-LPC were purchased from PerkinElmer Life Sciences. Nonradioactive fatty acids were obtained from Nu-Chek Prep. *sn*-1–16:0 LPC and *sn*-1–16:1 LPC were purchased from Avanti Polar lipids. CoA trilithium salt, acyl-CoA synthetase, and DAG were obtained from Sigma. [$^{14}$C]Acyl-CoAs were prepared according to the method described by Taylor *et al.* (15). *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC were prepared according to the assay used for measuring the forward reaction of LPCAT as described by Lager *et al.* (13) (see the details under "*in vitro* enzyme assays" for LPCAT forward reaction).

*Identification and Isolation of LPCAT Genes*—BLAST (16) searches were performed against the flax genome (17) and expressed sequence tag (EST) (14) databases by using AtLPCAT1 (At1G12640) and AtLPCAT2 (At1G63050) as the protein queries. The *LPCAT* genes were isolated according to gene isolation and cloning methods described in Pan *et al.* (5) and then subcloned into a pYES2.1/V5-His-TOPO vector using pYES2.1 TOPO kit (Invitrogen). The plasmids are referred to as pYESTOPO-*LPCATs*.

*Dating the Segmental Duplication Events*—To date the segmental duplication events, the $K_S$ values (synonymous substitution rates) of the duplicated genes, and 20 homologous gene pairs from their upstream and downstream flanking regions were used to calculate the average $K_S$. The full-length cDNA sequences of the homologous gene pair were first aligned based on their corresponding amino acid translations using Translator X server (18). Then the resulting alignment was further subjected to PAL2NAL (19) to calculate the corresponding $K_S$ value. Only $K_S$ values between 0 and 1 were included. The approximate date of the duplication event ($T$) was calculated using the equation $T = K_S/2\lambda$ (20), where the synonymous mutation rate per base ($\lambda$) of $1.5 \times 10^{-8}$ or $8.1 \times 10^{-9}$ is assumed (17) and the mean $K_S$ values from 21 homologous gene pairs is used.

*Plasmid Construction*—Plasmids used in our study are listed in Table 1. The integrity of all constructs was confirmed by sequencing.

A pair of duplicated *DGAT1* genes was previously identified in flax genome by our group (5). We cloned one of the two *DGAT1s*, named as *DGAT1-1*, into the pYESBOP vector as described in our previous study (5). In this study we cloned the *DGAT1-1* gene into the multiple cloning site 2 (MCS2) of pESC-URA for expression from the GAL1 promoter ($P_{GAL1}$). The resulting plasmid is referred as pECS-*DGAT1-1*. Next, the region from CYC1 terminator ($T_{CYC1}$) to ADH1 terminator of pESC-URA or pESC-*DGAT1-1* was amplified by PCR, and the resulting products were referred as fragment 1. At the same time, the entire plasmid, pYESTOPO-*LPCAT1* or pYESBOP, was also amplified by PCR, and the resulting PCR products were referred to as fragment 2. After the PCR amplification, fragment 1 was cloned into fragment 2 using the one-step, isothermal assembly method described by Gibson (21). Four different combinations of fragment 1 with fragment 2 resulted in four different plasmids, which are referred to as follows: 1) p*LPCAT1*+*DGAT1-1* contains both *LPCAT1* and *DGAT1-1* genes, and both genes are under $P_{GAL1}$; 2) p*LPCAT1* contains only the *LPCAT1* gene under $P_{GAL1}$; 3) p*DGAT1-1* contains only the *DGAT1-1* gene under $P_{GAL1}$; 4) pCTR is the control plasmid.

To construct the plasmid (p*FAD2*+*FAD3*+*HPH*) containing the replacement cassette for yeast mutant generation, previously isolated flax *FAD2–1* and flax *FAD3B* genes (5) were first amplified using PCR with the gene-specific primer pairs and then inserted into MSC2 of the pESC-URA, yielding pESC-*FAD2* and pESC-*FAD3*. Next, the cassettes $P_{GAL1}$-*FAD2*-$T_{CYC1}$ of pESC-*FAD2*, $P_{GAL1}$-*FAD3*-$T_{CYC1}$ of pESC-*FAD3*, and the hygromycin B resistance gene *hph* (hygromycin B phosphotransferase gene) from the plasmid pAG32 (22) were amplified by PCR and then inserted into the same pYESBOP plasmid using XhoI, EcoRI, and HindIII restriction sites, respectively. The resulting plasmid contained flax *FAD2–1* and *FAD3B* genes under the GAL1 promoter and the selectable marker gene *hph*.

*Yeast Strains*—Yeast strains used in this study are listed in Table 2.

Two types of yeast mutant strains (H1246 Δ *ale1* and H1246 *ale1* Δ::*FAD2*+*FAD3*) were constructed from the quadruple mutant strain *Saccharomyces cerevisiae* H1246, in which all four TAG biosynthesis genes (*dga1*, *lro1*, *are1*, and *are2*) are disrupted (23). H1246 Δ *ale1 is* a quintuple mutant strain. Besides the disrupted four genes in H1246, the *YOR175c* gene (also referred as *ALE1*) encoding LPCAT (24) was further deleted by using a disruption cassette containing a gene confer-

**TABLE 1**
**Plasmids used in this study**

| Plasmid name | Description | Reference |
|---|---|---|
| pYESTOPO-*LPCAT1* | pYES-$P_{GAL1}$-*LPCAT1*-$T_{CYC1}$ | This study |
| pYESTOPO-*LPCAT2* | pYES-$P_{GAL1}$-*LPCAT2*-$T_{CYC1}$ | This study |
| pYES-*DGAT1–1* | pYES-$P_{GAL1}$-*DGAT1–1*-$T_{CYC1}$ | (5) |
| pECS-*DGAT1−1* | pESC-$P_{GAL1}$-*DGAT1–1*-$T_{CYC1}$ | This study |
| pESC-*FAD2* | pESC-$P_{GAL1}$-*FAD2–1*-$T_{CYC1}$ | This study |
| pESC-*FAD3* | pESC-$P_{GAL1}$-*FAD3B*-$T_{CYC1}$ | This study |
| p*FAD2+FAD3+HPH* | pYES-$P_{GAL1}$-*FAD2–1*-$T_{CYC1}$/$P_{GAL1}$-*FAD3B*-$T_{CYC1}$/$P_{TEF1}$-*hphMX* | This study |
| p*LPCAT1+DGAT1–1* | pYES-$P_{GAL1}$-*LPCAT1*-$T_{CYC1}$/$P_{GAL1}$-*DGAT1–1*-$T_{CYC1}$ | This study |
| p*LPCAT1* | pYES-$P_{GAL1}$-*LPCAT1*-$T_{CYC1}$/$P_{GAL1}$-$T_{CYC1}$ | This study |
| p*DGAT1–1* | pYES-$P_{GAL1}$-$T_{CYC1}$/$P_{GAL1}$-*DGAT1–1*-$T_{CYC1}$ | This study |
| pCTR | pYES-$P_{GAL1}$-$T_{CYC1}$/$P_{GAL1}$-$T_{CYC1}$ | This study |
| pAG32 | pAG32-$P_{TEF1}$-*hphMX* | (22) |

**TABLE 2**
**Yeast Strains used in this study**

| Strain name | Genotype | Description | Reference |
|---|---|---|---|
| Y02431 (*ale1*Δ) | *Mat a, ale1*Δ, *his3*Δ*1, leu2*Δ*0, met15*Δ*0, ura3*Δ*0, YOR175c*::*KanMX4* | The *YOR175c* gene (also referred as *ale1*) encoding LPCAT is disrupted | (24) |
| H1246 | *Mat α, are1*-Δ::*HIS3, are2*-Δ::*LEU2, dga1*-Δ::*KanMX4, lro1*-Δ::*TRP1, ADE2* | Four genes *dga1*, *lro1*, *are1*, and *are2* encoding DGAT1, PDAT1, ASAT1, and ASAT2 are disrupted | (23) |
| H1246 Δ *ale1* | *Mat α, are1*-Δ::*HIS3, are2*-Δ::*LEU2, dga1*-Δ::*KanMX4, lro1*-Δ::*TRP1, ale1*-Δ::*HPH, ADE2* | H1246 in which *ale1* is deleted by using a destruction cassette containing a gene conferring resistance to hygromycin B (*hph*) | This study |
| H1246 *ale1* Δ::*FAD2+FAD3* | *Mat α, are1*-Δ::*HIS3, are2*-Δ::*LEU2, dga1*-Δ::*KanMX4, lro1*-Δ::*TRP1, ale1*Δ::*HPH + FAD2-1 + FAD3B, ADE2* | H1246, in which *ale1* is replaced by flax *FAD2–1* and *FAD3B* genes using a replacement cassette harboring genes encoding FAD2–1, FAD3B, and HPH | This study |

ring resistance to hygromycin B (*hph*). Another yeast mutant strain, H1246 *ale1* Δ::*FAD2+FAD3*, was created by replacing the *ale1* coding region with the gene replacement cassette containing genes encoding flax FAD2–1, flax FAD3B, and hygromycin B phosphotransferase .

A PCR-mediated gene disruption strategy (25) was used for yeast mutant construction. Using pAG32 and p*FAD2+FAD3+HPH* as templates, the *ale1* gene disruption and replacement cassettes were amplified by PCR, and the resulting PCR products were purified and then introduced into H1246 yeast cells using a standard lithium-acetate/PEG/single-strand DNA yeast transformation method (26) with slight modifications. Transformants were selected on YAPD plates containing hygromycin B (300 μg/ml) (Invitrogen). The correct disruption or replacement of the *ale1* gene in the mutants was verified by PCR with the *ale1* gene-specific and disruption cassette-specific/replacement cassette-specific verification primers designed according to Hegemann and Heick (27). The disruption of the *ale1* gene was further confirmed by performing the lyso-platelet-activating factor (lyso-PAF) sensitivity tests (the detailed procedure can be found under "Lyso-PAF sensitivity test" below).

*Heterologous Expression in Yeast*—In general, all yeast transformations were performed according to the standard lithium-acetate/PEG/single-strand DNA yeast transformation method (26). Transformants were screened on minimal medium plates lacking uracil (medium composition: 0.67% (w/v) yeast nitrogen base, 0.19% (w/v) synthetic complete medium lacking uracil (SC-Ura), 2% (w/v) dextrose, and 2% (w/v) agar) and confirmed by colony PCR. The recombinant yeast cells were first cultivated in liquid minimal medium containing 0.67% (w/v) yeast nitrogen base, 0.19% (w/v) SC-Ura, and 2% (w/v) raffinose and then grown in minimal medium containing 0.67% (w/v) yeast nitrogen base, 0.19% (w/v) SC-Ura, 2% (w/v) galactose, and 1% (w/v) raffinose (referred as induction medium) for induction of gene expression.

*Lyso-PAF Sensitivity Test*—A Lyso-PAF sensitivity test was performed using the method described in Chen *et al.* (24) with slight modifications. Yeast mutant strain Y02431 (*ale1*Δ) transformed with flax *LPCATs* was first grown in liquid minimal medium (lacking uracil) supplemented with 2% glucose overnight at 30 °C with shaking. After washing with sterile water 3 times, the overnight cultures were transferred to induction medium for 12 h. Cultures were then serial-diluted 1:10 from $A_{600\,nm} = 2$. A measure of 5 μl from each dilution was inoculated on minimal medium plates (lacking uracil) containing 0, 5, 10, and 20 μg/ml lyso-PAF. Yeast H1246 and Y02431 mutants transferred with empty vector pYES-*LacZ* were used as positive and negative controls, respectively. The plates were incubated at 28 °C for 2 days.

*The in Vivo Experiment*—The yeast mutant strain (H1246 *ale1* Δ::*FAD2+FAD3*) was used for *in vivo* studies. In the heterologous yeast expression system, an increased production of unsaturated fatty acids at low growth temperatures has been reported for many higher plant desaturases (28–30). For this reason, yeast mutant cells transformed with p*LPCAT1+DGAT1-1* or p*DGAT1-1* were grown at 20 °C in induction medium. After 3 days, the cells were harvested and used for yeast lipid analysis. Yeast lipid extraction and analysis was performed according to the method described in Pan *et al.* (5).

*The in Vitro Experiments*—Microsomal preparations were performed according to the method described in Pan *et al.* (5). The crude protein concentration in the microsomal preparations was measured using the Bio-Rad Bradford assay (Bio-Rad). Before the enzymatic assays, the effect of protein concentration, incubation time, and substrate concentrate on enzyme

activity was evaluated to optimize enzymatic reaction conditions for measuring substrate specificities of the DGAT- and LPCAT-catalyzed forward reactions.

*DGAT Enzyme Assays*—The DGAT enzyme assays were performed using the method described by Byers *et al.* (31), with some modifications. Microsomal preparations from H1246 cells transformed with pYES-*DGAT1-1* were used in the assays. Control microsomes were prepared from H1246 yeast transformed with pYES-*LacZ*. The reaction mixture contained 5 $\mu$g of microsomal protein, 200 mM HEPES-NaOH, pH 7.4, 3.2 mM $MgCl_2$, 333 $\mu$M *sn*-1,2-diolein, and 15 $\mu$M [$^{14}$C]acyl-CoA in a total volume of 60 $\mu$l. The reaction was incubated at 30 °C for 6 min and then quenched by the addition of 10 $\mu$l of sodium dodecyl sulfate 10% (w/v). The reaction was then spotted on a silica thin layer chromatography (TLC) plate (SIL G25, 0.25 mm, Macherey-Nagel, Germany). The plate was developed with hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Corresponding TAG spots were scraped from the plate, and radioactivity was measured by a LS 6500 multi-purpose scintillation counter (Beckman-Coulter).

*LPCAT Enzyme Assays*—The LPCAT enzyme assays were performed according to the method previously described by Lager *et al.* (13) with slight modifications. Microsomes were prepared from the yeast mutant strain Y02431 (*ale1*Δ) transformed with pYESTOPO-*LPCAT1*. Microsomes prepared from yeast containing pYES-*LacZ* were used as a negative control.

For testing the forward activity, the reaction mixture contained 0.3 $\mu$g of microsomal protein, 200 $\mu$M [$^{14}$C]18:1-LPC, 100 $\mu$M acyl-CoA, and 0.1 M potassium phosphate buffer, pH 7.2, in a total volume of 50 $\mu$l. The reaction was incubated at 30 °C for 5 min and then terminated by the addition of 170 $\mu$l of 5% acetic acid and 500 $\mu$l of chloroform/methanol (1:1, v/v). The mixture was extracted and then applied to a TLC plate. The TLC plate was developed with a solvent system of chloroform/methanol/formic acid (75:25:10, v/v/v). Radioactivity of the PC spots was determined by scintillation counting.

For measuring the reverse activity, an aliquot of microsomal protein (43 $\mu$g, which equals ~10 nmol of endogenous PC) was lyophilized overnight. The substrate, 9 nmol of *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC (2.5 nCi/nmol), was dissolved in 14 $\mu$l of benzene and applied directly to the freeze-dried microsomes. The benzene was dried immediately under $N_2$ at 37 °C, after which 100 $\mu$l of reaction mixture containing 200 nmol of free CoA, 10 nmol of 18:1-CoA, and 1 mg of bovine serum albumin (BSA) in 0.1 M potassium phosphate buffer, pH 7.2, was added. The assay was incubated at 30 °C for 60 min and then quenched with 100 $\mu$l of 0.15 M acetic acid and 500 $\mu$l of chloroform/methanol (1:2, v/v). The mixture was then extracted with the Bligh and Dyer method (32). The [$^{14}$C]acyl-CoAs in the upper phase was further hydrolyzed to free fatty acids with KOH. After neutralization with HCl, the free fatty acids were extracted into chloroform. Radioactivity of $^{14}$C-labeled free fatty acids was determined by scintillation counting.

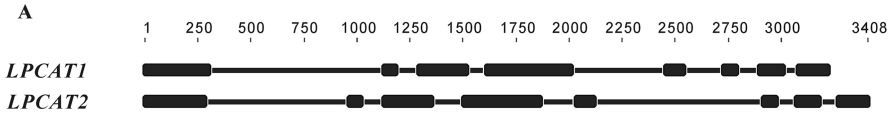
*LPCAT1 and DGAT1-1 Coupling Enzyme Assays*—The yeast quintuple mutant strain H1246 Δ *ale1* was used to test the coupling hypothesis under *in vitro* conditions. Microsomes were prepared from the cells transformed with p*LPCAT1*+*DGAT1-1*, p*LPCAT1*, or p*DGAT1-1*. Control microsomes were prepared from yeast cells transformed with pCTR. A 43-$\mu$g portion of microsomal preparations was lyophilized overnight. The substrates of 9 nmol of *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC (2.5 nCi/nmol) and 32 nmol of *sn*-1,2-diolein were dissolved in 14 $\mu$l of benzene and applied directly to the freeze-dried microsomes. The benzene was dried immediately under $N_2$ at 37 °C. The assay was started by adding 100 $\mu$l of reaction mixture containing 200 nmol of free CoA, 10 nmol of 18:1-CoA, and 1 mg of BSA in 0.1 M potassium phosphate buffer, pH 7.2. After incubation for 60 min at 30 °C, the assay was terminated by the addition of 100 $\mu$l of 0.15 M acetic acid and 500 $\mu$l of chloroform/methanol (1:2, v/v). Lipids were extracted into chloroform and then applied to the TLC plate. The resulting TLC plate was first developed in a solvent system of chloroform/methanol/acetic acid/$H_20$ (60:30:3:1, v/v/v/v) to half-plate. After drying in a $N_2$-filled chamber, the plate was then developed with a solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v) to full plate. The corresponding TAG spots were scraped, and radioactivity was measured by scintillation counting. The coupling activities were determined as the amount of [$^{14}$C]TAG produced.

## Results

As a prelude to investigating possible coupling of the LPCAT-catalyzed reaction to the DGAT1-catalyzed reaction, we first examined the properties of individual flax enzymes.

*Identification and Isolation of LPCAT Genes from Flax*—Through exploring flax genome and EST databases, we identified a pair of duplicated *LPCAT* genes in flax. Both *LPCAT* genes have a coding region of 1380 bp and encode proteins with 460-amino acid residues. These two LPCATs share 96.3% and 96.5% identity at the nucleotide and amino acid level, respectively. Both *LPCAT* genes contain eight exons and seven introns (Fig. 3*A*). Further genomic analysis revealed that the protein-coding genes flanking the *LPCAT* genes are highly conserved, suggesting that the duplicated *LPCAT* genes were derived from a segmental duplication event. In addition, the mean $K_S$ value of 21 homologous gene pairs (including the *LPCAT* gene pair) within the segmental duplication blocks was used as a proxy of time to estimate the date of the segmental duplication event. The result presented in Fig. 3*B* shows that a segmental duplication event occurred ~5.1–9.5 million years ago (MYA), which is consistent with the time of a recent (5–9 MYA) whole-genome duplication (WGD) in flax (17), suggesting that the segmental duplication is the result of a recent WGD event.

*LPCAT cDNAs from Flax Encode Functionally Active LPCAT Enzymes*—To determine whether LPCAT cDNAs from flax encode proteins possessing LPCAT activity, the cDNAs of both *LPCATs* were amplified from flax embryos and cloned into the yeast expression pYES2.1TOPO vector. The resulting recombinant plasmids were transformed into the yeast mutant strain Y02431 (*ale1*Δ), which has a very low LPCAT background activity (Chen *et al.*; Ref. 24). After transformation, a lyso-PAF sensitivity test was performed to confirm the proper expression of *LPCAT* genes in yeast mutant Y02431. Yeast H1246 and Y02431 mutants containing pYES-*LacZ* were used as positive and negative controls, respectively. The result (Fig. 3*C*) showed




B

| Segment Pairs | Number of gene Pairs included | *Ks* (Mean±SE) | Estimated time (MYA) |
|---|---|---|---|
| *LPCAT* | 21 | 0.154±0.077 | 5.1 - 9.5 |
| *DGAT1* | 21 | 0.148±0.109 | 4.9 - 9.2 |

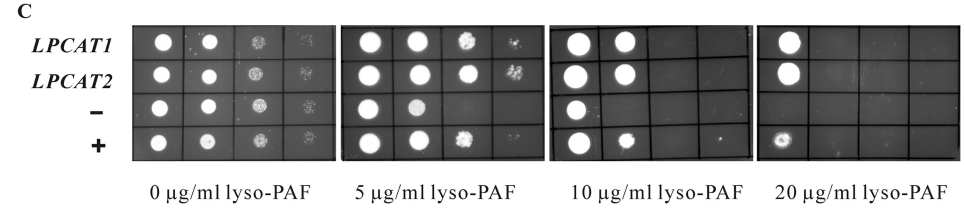



FIGURE 3. **Results obtained from gene structure analyses, estimation of gene duplication dates, and lyso-PAF sensitivity tests.** *A*, genomic DNA structure of flax *LPCATs*. The *thick lines* represent exons, and the *thin lines* represent introns. The *numbers on the top* denote the scale in base pairs. *B*, the mean $K_S$ values and estimated dates for segmental duplication events corresponding to flax *LPCAT* and *DGAT1* genes. *C*, lyso-PAF sensitivity tests of yeast mutant Y02431 expressing flax *LPCATs*. Yeast H1246 and Y02431 mutant strains containing empty vector (pYES-*LacZ*) were used as positive (+) and negative (−) controls, respectively. Cultures were serial-diluted 1:10 from $A_{600\ nm} = 2$. Four concentrations of lyso-PAF (0, 5, 10, 20 μg/ml) were tested. A detailed procedure is described under "Experimental Procedures." $K_S$, synonymous substitution rate; *MYA*, million years ago.

that there was a severe growth defect in yeast mutant Y02431 transformed with pYES-*LacZ* when the plate was supplemented with 10 μg/ml lyso-PAF. No growth was observed for the negative control when 20 μg/ml lyso-PAF were added into the plates. However, yeast cells expressing *LPCAT1* or *LPCAT2* were able to overcome the growth inhibition of lyso-PAF and exhibited a growth even better than the vector-only H1246 transformants in the presence of 5, 10, and 20 μg/ml lyso-PAF. These results confirmed that these recombinant yeast mutant Y02431 can be further used for enzyme assays. Given our previous finding that the recently duplicated genes in flax, such as *DGAT2-1/DGAT2-3* and *PDAT1/PDAT5*, exhibit similar gene expression patterns and encode proteins with similar substrate selectivity (5), only one of the duplicated *LPCAT* genes, *LPCAT1*, was used for subsequent analyses.

*Flax LPCAT1 Shows High Acyl Substrate Specificity toward PUFA-containing Substrates in Both the Forward and Reverse Reactions*—To further assess LPCAT enzymatic properties, the acyl substrate specificities of LPCAT were evaluated in the forward and reverse reactions. We first assessed the preference of LPCAT1 toward various acyl-CoA donors in the forward reaction (Fig. 4*A*). LPCAT1 exhibited a higher preference for unsaturated fatty acyl-CoAs, including 18:1, 18:2, and 18:3, over the saturated fatty acyl-CoAs. The highest activity was observed with PUFA-CoAs (18:2- and 18:3-CoAs). To measure the acyl specificity in the reverse reaction, different *sn*-2-[$^{14}$C]acyl-PC species (*sn*-2-[$^{14}$C] 18:1-, 18:2-, and 18:3-PC) were used as substrates. Among the tested substrates, the reverse reaction of LPCAT showed significantly higher specificity toward *sn*-2-[$^{14}$C] 18:2- and 18:3-PC than toward 18:1-PC (Fig. 4*B*). In contrast to the previously reported *Arabidopsis* LPCAT (13) with relatively low preference for *sn*-2–18:2-PC, LPCAT from flax transferred 18:2 acyl groups from PC at the highest rate. Together, these results suggested that both forward and reverse reactions prefer PUFA-containing substrates.

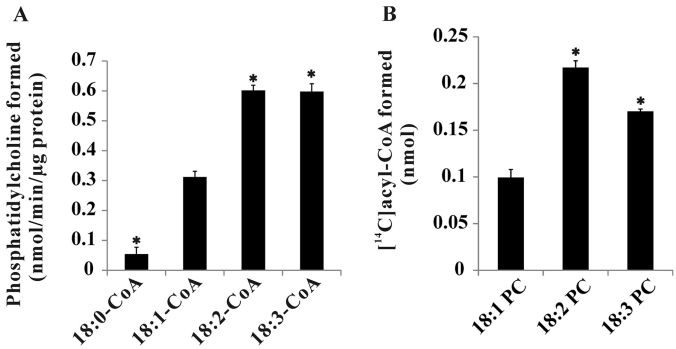



FIGURE 4. **Acyl substrate specificities of flax LPCAT1 in the forward (*A*) and reverse (*B*) reactions.** Microsomal preparations from the yeast mutant strain Y02431 (*ale1*Δ) expressing flax *LPCAT1* were used in assays. For measuring acyl specificities in the forward reaction, different unlabeled acyl-CoAs and [$^{14}$C]18:1-LPC were used as substrates. For measuring acyl specificities in the reverse reaction, different *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC species were provided as substrates. Detailed procedures for measuring the forward and reverse activities of LPCAT are described under "Experimental Procedures." Data are represented as the mean of duplicate assays ± S.E. The *asterisks* indicate significant differences to 18:1-CoA or 18:1-PC (analysis of variance, least significant difference test) at $p < 0.05$ level.

*Flax DGAT1-1 Displayed Small but Significantly Enhanced Specificity for 18:3-CoA*—We previously identified a pair of *DGAT1* genes with 97.7% sequence identity in the flax genome (5). $K_S$ analysis showed that similar to *LPCATs*, the duplicated *DGAT1*s were derived from a recent whole-genome duplication (Fig. 3*B*). Our previous study (5, 33) showed that flax DGAT1-1 was able to complement the TAG synthesis deficiency in yeast mutant strain H1246, in which all four TAG biosynthesis genes are disrupted. Experiments of *in vivo* fatty acid feeding indicated that DGAT1-1 produced a similar amount of TAG in the presence of diverse, exogenously provided fatty acids, suggesting that DGAT1-1 may have a broad substrate preference (5). In this study, we further examined fatty acyl-CoA substrate specificity for flax DGAT1-1 under *in*

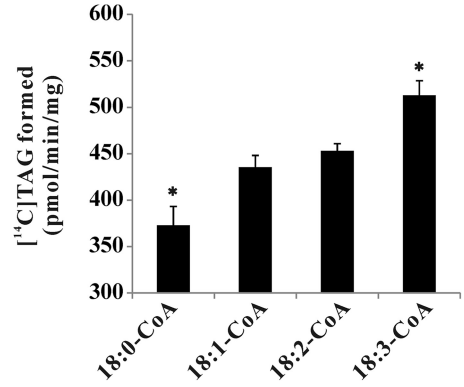



FIGURE 5. **Acyl substrate specificities of flax DGAT1-1.** Microsomal preparations from the yeast mutant H1246 expressing flax *DGAT1-1* were incubated with different [$^{14}$C]acyl-CoAs and unlabeled *sn*-1,2-diolein. Data are represented as the mean of triplicate assays ± S.E. The *asterisks* indicate significant differences to 18:1-CoA (analysis of variance, least significant difference test) at $p < 0.05$ level.

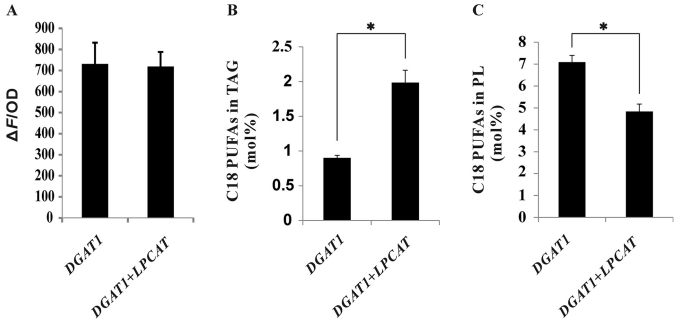



FIGURE 6. ***In vivo* evidence supporting the biochemical coupling of the flax LPCAT1-catalyzed reverse reaction with the flax DGAT1-1-catalyzed reaction.** *A*, Nile red assays performed with yeast cells co-expressing flax *DGAT1-1* with *LPCAT1* or expressing *DGAT1-1* alone. The values represent the amount of TAG accumulated in the recombinant yeast cells, which is calculated by dividing Nile red fluorescence (Δ*F*) by the optical density (*OD*) at 600 nm. *B*, mole percentage of 18C PUFAs in TAG of the recombinant yeast cells. *C*, mole percentage of 18C PUFAs in phospholipid (*PL*) of the recombinant yeast cells. Instead of exogenous fatty acid feeding, 18C PUFAs were synthesized by co-expressing flax *FAD2-1* and *FAD3B* genes in yeast mutant strains. Data are represented as the mean (five biological replicates) ± S.E. The *asterisks* indicate significant differences in 18C PUFA levels of the TAG and PL fractions from yeast cells co-expressing flax *DGAT1-1* with *LPCAT1 versus* expressing *DGAT1-1 alone* (analysis of variance, least significant difference test) at $p < 0.05$ level.

*vitro* conditions. The same yeast mutant strain, H1246, was used because it provides a clean background to detect [$^{14}$C]TAG produced from the introduced flax DGAT1-1. The assays were carried out with microsomal preparations from the H1246 mutant expressing flax *DGAT1-1*. Different [$^{14}$C]acyl-CoAs were provided along with nonradiolabeled *sn*-1,2-dioleoylglycerol. As shown in Fig. 5, DGAT1-1 exhibited small but significant changes of substrate specificity toward the acyl-CoA substrates, and the highest specificity was observed with 18:3-CoA, which is in close agreement with substrate specificity data for overall microsomal DGAT activity from developing flax embryos (34).

*In Vivo Evidence of Biochemical Coupling of LPCAT1 and DGAT1-1-catalyzed Reactions*—After enzymatic characterization of individual flax LPCAT1 and DGAT1-1 enzymes, we started testing the coupling hypothesis in a yeast system under *in vivo* conditions. To avoid interference from endogenous TAG-synthesizing and LPCAT activities, we constructed a new yeast mutant strain from H1246. In the newly generated mutant strain, referred to as H1246 *ale1* Δ::*FAD2*+*FAD3* mutant strain (Table 2), the endogenous *ale1* gene was replaced by genes encoding FAD2-1 and FAD3B from flax; thus, no significant TAG-synthesizing and LPCAT activities were present in the mutant. The rationale behind inserting *FAD2* and *FAD3* into the yeast chromosome is that: 1) 18:2 and 18:3 do not naturally occur in the H1246 mutant, and 2) FAD2 and FAD3 catalyze the synthesis of 18:2 and 18:3, respectively, on the *sn*-2 position of PC, which is also the major substrate for the LPCAT reverse reaction. The newly created yeast strain containing flax *FAD2–1* and *FAD3B* genes can synthesize 18:2 and 18:3 on the *sn*-2 position of PC, and in this way, we avoided exogenous fatty acid feeding, which usually gives a random distribution of fatty acids over all *sn* positions in all types of lipid classes. Constructs containing flax *DGAT1-1* alone or both *DGAT1-1* and *LPCAT1* were then individually introduced into this newly generated yeast strain. We hypothesized that the reverse action of LPCAT can catalyze the transfer of PUFAs produced on PC directly into the acyl-CoA pool, making the PUFA-CoAs available for the DGAT1-catalyzed reaction for TAG production. Consistent with our hypothesis, whereas no significant differences in the total amount of TAG were found between yeast cells expressing flax *DGAT1-1* and yeast cells co-expressing flax *DGAT1-1* and *LPCAT1* (Fig. 6*A*), co-expressing flax *DGAT1-1* and *LPCAT1* significantly increased 18-carbon PUFAs (18:2 and 18:3) in TAG with a concomitant decrease of 18-carbon PUFAs in phospholipid, as opposed to those expressing *DGAT1-1* only (Fig. 6, *B* and *C*).

*In Vitro Evidence of Biochemical Coupling of LPCAT1 and DGAT1-1 Reverse Reactions*—Next, we tested the coupling hypothesis under *in vitro* conditions. For this purpose, we constructed another quintuple yeast mutant, referred as H1246 Δ *ale1* (Table 2), in which the endogenous *ale1* gene of H1246 was disrupted. The newly generated quintuple mutant was unable to synthesize TAG and had a low background LPCAT activity. The mutant was then transformed with p*LPCAT1*+*DGAT1-1*, p*LPCAT1*, p*DGAT1-1*, or pCTR, individually. After induction, microsomes of the recombinant yeast cells were isolated and fed with PC radiolabeled with $^{14}$C at the *sn*-2 position. We hypothesized that the biochemical coupling can enhance the transfer of $^{14}$C-labeled acyl groups from PC into TAG via the reverse reaction of LPCAT1 coupled to the DGAT1-1-catalyzed reaction. As shown in Fig. 7, *A* and *B*, [$^{14}$C]TAG was formed when *sn*-2-[$^{14}$C] acyl-PC (*sn*-2-[$^{14}$C] 18:1-PC, *sn*-2-[$^{14}$C] 18:2-PC, or *sn*-2-[$^{14}$C] 18:3-PC) was incubated with yeast microsomes containing recombinant flax LPCAT1 and flax DGAT1-1. In contrast, no [$^{14}$C]TAG was detected after incubation of the radiolabeled PC with microsomes containing either flax LPCAT1 or flax DGAT1-1 alone. In addition, no [$^{14}$C]TAG could be detected when incubations were conducted with control microsomes. This result confirmed our hypothesis. It should be noted that, as shown in Fig. 7*A*, the assays also produced $^{14}$C-free fatty acids, which may arise from the hydrolysis of [$^{14}$C]acyl-CoAs catalyzed by the endogenous acyl-CoA thioesterase (13).

We further tested the acyl specificities in overall LPCAT1 and DGAT1-1 coupling process by presenting *sn*-2-[$^{14}$C] 18:1-,

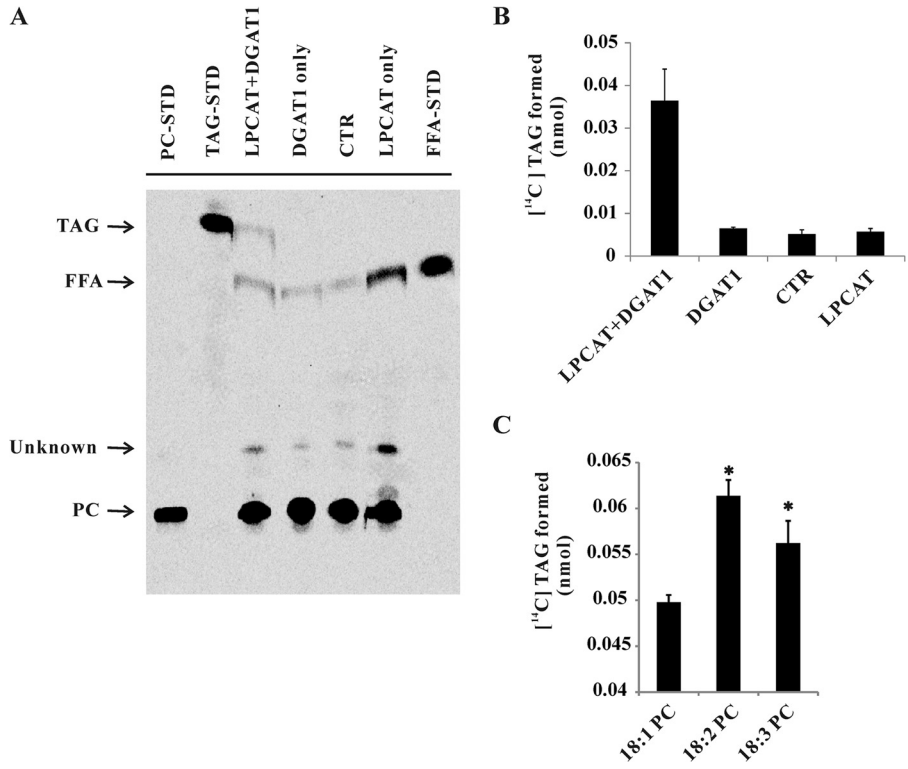



FIGURE 7. ***In vitro* evidence supporting the biochemical coupling of the flax LPCAT1-catalyzed reverse reaction with the flax DGAT1-1-catalyzed reaction.** *A*, a representative TLC plate of flax LPCAT1 and DGAT1-1 coupling enzyme assay. Microsomal preparations from the yeast quintuple mutant H1246 Δ *ale1* individually transformed with p*LPCAT1*+*DGAT1-1*, p*LPCAT1*, p*DGAT1-1*, or pCTR were incubated with *sn*-1–16:0-*sn*-2-[$^{14}$C]18:1-PC and unlabeled *sn*-1,2-diolein. Followed by the enzyme assay, the lipid fractions were extracted and separated by TLC plates. Radiolabeled [$^{14}$C] TAG was only formed with yeast microsomes containing both flax LPCAT1 and DGAT1-1. *B*, the amount of [$^{14}$C]TAG produced in the recombinant yeast cells. Data are represented as the means of triplicate assays ±S.E. *C*, the specific activity of overall DGAT1-1 and LPCAT1 coupling process. Different *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC species along with unlabeled *sn*-1,2-diolein were used as substrates. Data are represented as the mean of triplicate assays ± S.E. The *asterisks* indicate significant differences to 18:1-PC (analysis of variance, least significant difference test) at $p < 0.05$. *FFA*, free fatty acid; *STD*, standard; *CTR*, control (yeast cells transformed with pCTR plasmid).

18:2-, or 18:3- PC as single substrates to the microsomes containing both flax LPCAT1 and flax DGAT1-1. As shown in Fig. 7*C*, yeast microsomes containing flax LPCAT1 and flax DGAT1-1 were able to transfer the $^{14}$C-labeled 18:2 or 18:3 moiety at a higher rate than 18:1 moiety from the *sn*-2 position of PC to TAG, which was consistent with our *in vivo* data.

## Discussion

Using acyl-CoAs as donors, two major biochemical pathways, the Kennedy pathway (*de novo* pathway) (35) and the Lands cycle (remodeling pathway) (10), contribute to PC biosynthesis (Fig. 1). Both pathways were proposed in 1950s. PC serves not only as a major component of cellular membrane but also as the primary site for PUFA or unusual fatty acid biosynthesis. The accumulation of a large proportion of PUFAs in flax seed oils necessitates efficient mechanisms to channel PUFAs from PC into TAG. Our previous studies have shown that PDAT-mediated TAG biosynthesis (5) and phosphatidylcholine:diacylglycerol cholinephosphotransferase-based PC-DAG interconversion (36) play critical roles in regulating PUFA content in flax seed oil. The Kennedy pathway and the Lands cycle are connected by LPCAT-catalyzed reactions. Here, we hypothesize that the reverse reaction of LPCAT can be coupled to the DGAT1 reaction to produce the PUFA-containing TAG.

Our hypothesis is supported by the following observations. 1) Acyl specificity tests showed that the reverse reaction of flax LPCAT1 has the highest preference toward PUFA-containing PC (Fig. 4*B*). In addition, the flax DGAT1-1 forward reaction showed a small but significant preference for 18:3-CoAs (Fig. 5). 2) Under *in vivo* conditions, DGAT1-1 produced a higher amount of PUFA-containing TAG and a lower amount of PUFA-containing phospholipid in the presence than in the absence of LPCAT1 (Fig. 6, *B* and *C*). 3) Under *in vitro* conditions, when *sn*-1–16:0-*sn*-2-[$^{14}$C]acyl-PC was included as the substrate, radiolabeled [$^{14}$C]TAG was formed only in the reaction with the yeast microsomes containing both flax LPCAT1 and flax DGAT1-1 (Fig. 7, *A* and *B*). In contrast, under the same assay conditions, no [$^{14}$C]TAG was detected in reactions with microsomes containing either flax LPCAT1 or flax DGAT1-1 alone. These results provided strong evidence supporting our hypothesis. 4) The acyl group specificity of overall LPCAT1 and DGAT1-1 coupling process under *in vitro* conditions showed that yeast microsomes containing DGAT1-1 and LPCAT1 were able to transfer the $^{14}$C-labeled linoleoyl or linolenoyl moiety at a higher rate than the oleoyl moiety from the *sn*-2 position of PC to TAG (Fig. 7*C*).

Given that PUFA groups could be deacylated by the action of PLA on PC and previous work (37) indicated that the expression of $PLA_2$ can be affected by altered expression of *LPCAT*, there was the possibility that expression of *LPCAT* triggered the expression of $PLA_2$, which could contribute to the increased

PUFA content in TAG. It is important to note that PLA-catalyzed deacylation of PC produces free fatty acids. Before they can be used by the DGAT reaction, free fatty acids need to be activated to acyl-CoAs. The formation of the acyl-CoAs is catalyzed by acyl-CoA synthetase, which requires ATP for its action. However, the assay mixture used in this study did not contain ATP. More importantly, we failed to detect any [$^{14}$C]TAG products in the reaction with microsomes containing flax DGAT1-1 alone (Fig. 7, *A* and *B*) indicating that under our assay conditions, there was little or no interference from PLA. Interference may also arise from PC to DAG conversion catalyzed by the phospholipase C reaction or a combined reaction of phospholipase D/phosphatidic acid phosphatase. The resulting PUFA-containing DAG may be further incorporated into TAG by the DGAT-catalyzed reaction and lead to an increased content of PUFAs in TAG. For the same reason described above, this interference was negligible because we did not detect any [$^{14}$C]TAG when microsomes contained only recombinant flax DGAT1-1. Taken together, our data support the hypothesis that the transfer of PUFAs formed on PC to the acyl-CoA pool and further incorporated into TAG is at least to some extent catalyzed through coupling between the reverse reaction of flax LPCAT1 and the forward reaction of flax DGAT1-1.

It was proposed nearly 30 years ago that LPCAT can catalyze both forward and reverse reactions in plants (9). However, equilibrium of the LPCAT reaction favors the exothermic forward reaction. It was not until recently that Stymne and coworkers (13) proved that plant LPCAT enzyme does in fact operate in a reversible fashion under *in vitro* conditions. An intriguing question remains: How does the endothermic reverse reaction take place *in vivo*? It was previously suggested that for the LPCAT reverse reaction to take place *in vivo*, the PC content and free CoA has to be very high in compared with the LPC content and available acyl-CoA within the cell (13). Based on our data, we propose an alternative scenario as follows: under certain metabolic conditions, the endothermic LPCAT-catalyzed reverse reaction can be pulled via coupling it to the DGAT1-catalyzed forward reaction, which is strongly exothermic and has a shared intermediate, acyl-CoA. The effective removal of acyl-CoAs from the acyl-CoA pool catalyzed by DGAT1 can lower the concentration of acyl-CoA and thus stimulate the LPCAT-catalyzed reaction to operate in the reverse direction. It should be noted that the LPCAT-catalyzed reaction is not a part of a linear biosynthetic pathway, but it is the combined forward and reverse reaction of the enzyme that catalyzes an exchange of acyl groups between PC and acyl-CoA pools where the acyl-CoA and LPC formed by the reverse reaction can be immediately re-acylated by the forward reaction. As shown in Fig. 4*A*, the forward reaction of flax LPCAT1 has highest specificities toward the PUFA-CoAs, indicating that once transferred to the acyl-CoA pool, PUFA groups can be quickly re-acylated into PC by the forward action of LPCAT. To maintain a sustained reverse reaction, the re-acylation of the formed LPC by the forward reaction of LPCAT has to occur simultaneously. In addition, the specific activity of flax LPCAT1-catalyzed forward reaction is ~1000 times higher than that of flax DGAT1-catalyzed reaction when the expressed recombinant enzymes were assessed in yeast microsomes under *in vitro* enzyme assays (Fig. 4*A* and Fig. 5). This forward reaction of LPCAT has the potential to compete with the DGAT1-1-catalyzed reaction for PUFA-CoA substrates. Therefore, to produce PUFA-enriched TAG, especially ALA-enriched TAG, the DGAT reaction has to be highly selective toward ALA-CoAs. It should be noted that even though flax DGAT1-1 showed enhanced substrate specificity toward ALA-CoAs, the differences in substrate specificity were fairly small (Fig. 5). Our previous *in vivo* fatty acid feeding experiments showed that recombinant flax DGAT2 appears to have a stronger preference than flax DGAT1-1 toward ALA-containing substrates (5). The activity of flax DGAT2 in the yeast system, however, was very low, which limits its potential use in testing the coupling hypothesis in this study. In the future it will be very interesting to find out whether using codon-optimized flax DGAT2 and flax LPCAT1 can further enhance the ALA content in TAG. The enhanced specificity of DGAT2 for ALA-CoA may provide a further operational advantage in the coupling process, and the extent of this coupling process could potentially be influenced by the fatty acyl composition of the acyl-CoA pool. Furthermore, LPCAT and DGAT enzymes might physically interact with each other in the membrane. If LPCAT is physically associated with DGAT1 *in vivo*, the products of the LPCAT-catalyzed reverse reaction, PUFA-CoAs, can be potentially channeled directly into DGAT, providing an enhanced efficiency of PUFA-CoA transfer and establishing a favorable operating condition for the reverse reaction of LPCAT. Thus, substrate selectivity and substrate channeling may work collectively to promote the incorporation of PUFAs into TAG while overcoming a formidable thermodynamic barrier.

It is well known that PC serves not only the primary site for PUFA biosynthesis but also as the major site for biosynthesis of a number of unusual fatty acids. Our results show that the reverse reaction of LPCAT1 can be coupled with the DGAT1 reaction to enhance PUFA content in TAG. The biochemical coupling mechanism found in flax might also come into play in other plant systems, such as castor, which accumulates oil enriched in the unusual fatty acid known as ricinoleic acid. It may be difficult, however, to test the hypothesis of biochemical coupling of castor LPCAT-catalyzed and DGAT-catalyzed reactions using a yeast system because of potential interference by endogenous thioesterase activity, which appears to readily catalyze the hydrolysis of hydroxyacyl-CoA (13). If the hydroxyacyl-CoA generated by the castor LPCAT-catalyzed reaction, however, is immediately used by the castor DGAT-catalyzed reaction and castor DGAT exhibits a high affinity for hydroxyacyl-CoA, then the endogenous thioesterase activity may not be an issue. Indeed, as indicated in the previous study, castor DGAT2 exhibits enhanced preference for hydroxyacyl-CoAs (38). The high activity of castor DGAT2 on hydroxyacyl-CoA may keep the free concentration of hydroxyacyl-CoA low enough to effectively pull LPCAT in the reverse direction. Further experiments are needed to confirm the biochemical coupling of LPCAT and DGAT in other plant species.

In conclusion, seed oils producing enhanced levels of PUFAs or unusual fatty acids have a wide range of food, feed, and industrial applications. Increasing our insight into the incorporation

of PC-modified fatty acids into TAG can provide new strategies to effectively modify seed oils for different uses. In this study we provide direct *in vivo* and *in vitro* evidence for biochemical coupling of the flax LPCAT1-catalyzed reverse reaction to the flax DGAT1-1-catalyzed reaction as a route for enhancing the amount of PUFA in TAG. A similar mechanism may apply to other plant species that accumulate PUFAs or unusual fatty acids in their oils. Our results provide an increased understanding of PC metabolism in relation to the Kennedy pathway for generating PUFA-enriched TAG and will benefit future initiatives aimed at enhancing the PUFA content of TAG in plants.

*Author Contributions*—X. P. planed and performed all experiments, carried out data analyses, and drafted the article. R. J. W. and X. P. designed the research. J. Z., G. C., M. K., M. S. G., and K. M. C. contributed valuable discussions during this study. All co-authors contributed to the revision of this work.

*Acknowledgments—We give special thanks to Dr. Sten Stymne (Swedish University of Agricultural Sciences) for valuable comments and discussions. We also thank Dr. Xiao Qiu for use of laboratory facilities at University of Saskatchewan in Saskatoon and Dr. Ida Lager (Swedish University of Agricultural Sciences) for providing suggestions on determining the reverse activity of LPCAT under in vitro* conditions.

## References


1. Ohlrogge, J., and Browse, J. (1995) Lipid biosynthesis. *Plant Cell* **7,** 957–970
2. Sperling, P., Linscheid, M., Stöcker, S., Mühlbach, H. P., and Heinz, E. (1993) *In vivo* desaturation of *cis*-Δ9-monounsaturated to *cis*-Δ9,12-diunsaturated alkenylether glycerolipids. *J. Biol. Chem.* **268,** 26935–26940
3. Carlsson, A. S., Yilmaz, J. L., Green, A. G., Stymne, S., and Hofvander, P. (2011) Replacing fossil oil with fresh oil: with what and for what? *Eur. J. Lipid Sci. Technol.* **113,** 812–831
4. Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., and Stymne, S. (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 6487–6492
5. Pan, X., Siloto, R. M., Wickramarathna, A. D., Mietkiewska, E., and Weselake, R. J. (2013) Identification of a pair of phospholipid: diacylglycerol acyltransferases from developing flax (*Linum usitatissimum* L.) seed catalyzing the selective production of trilinolenin. *J. Biol. Chem.* **288,** 24173–24188
6. Kim, H. U., Lee, K. R., Go, Y. S., Jung, J. H., Suh, M. C., and Kim, J. B. (2011) Endoplasmic reticulum-located PDAT1–2 from castor bean enhances hydroxy fatty acid accumulation in transgenic plants. *Plant Cell Physiol.* **52,** 983–993
7. van Erp, H., Bates, P. D., Burgal, J., Shockey, J., and Browse, J. (2011) Castor phospholipid:diacylglycerol acyltransferase facilitates efficient metabolism of hydroxy fatty acids in transgenic *Arabidopsis*. *Plant Physiol.* **155,** 683–693
8. Hu, Z., Ren, Z., and Lu, C. (2012) The phosphatidylcholine diacylglycerol cholinephosphotransferase is required for efficient hydroxy fatty acid accumulation in transgenic *Arabidopsis*. *Plant Physiol.* **158,** 1944–1954
9. Stymne, S., and Stobart, A. K. (1984) Evidence for the reversibility of the acyl-CoA:lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons and rat liver. *Biochem. J.* **223,** 305–314
10. Lands, W. E. (1958) Metabolism of glycerolipides: a comparison of lecithin and triglyceride synthesis. *J. Biol. Chem.* **231,** 883–888
11. Lands, W. E. (1960) Metabolism of glycerolipids. 2. The enzymatic acylation of lysolecithin. *J. Biol. Chem.* **235,** 2233–2237
12. Bates, P. D., Fatihi, A., Snapp, A. R., Carlsson, A. S., Browse, J., and Lu, C. (2012) Acyl editing and headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into triacylglycerols. *Plant Physiol.* **160,** 1530–1539
13. Lager, I., Yilmaz, J. L., Zhou, X. R., Jasieniecka, K., Kazachkov, M., Wang, P., Zou, J., Weselake, R., Smith, M. A., Bayon, S., Dyer, J. M., Shockey, J. M., Heinz, E., Green, A., Banas, A., and Stymne, S. (2013) Plant acyl-CoA: lysophosphatidylcholine acyltransferases (LPCATs) have different specificities in their forward and reverse reactions. *J. Biol. Chem.* **288,** 36902–36914
14. Venglat, P., Xiang, D., Qiu, S., Stone, S. L., Tibiche, C., Cram, D., Alting-Mees, M., Nowak, J., Cloutier, S., Deyholos, M., Bekkaoui, F., Sharpe, A., Wang, E., Rowland, G., Selvaraj, G., and Datla, R. (2011) Gene expression analysis of flax seed development. *BMC Plant Biol.* **11,** 74
15. Taylor, D. C., Weber, N., Hogge, L. R., and Underhill, E. W. (1990) A simple enzymatic method for the preparation of radiolabeled erucoyl-CoA and other long-chain fatty acyl-CoAs and their characterization by mass spectrometry. *Anal. Biochem.* **184,** 311–316
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410
17. Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L., Hawkins, S., Neutelings, G., Datla, R., Lambert, G., Galbraith, D. W., Grassa, C. J., Geraldes, A., Cronk, Q. C., Cullis, C., Dash, P. K., Kumar, P. A., Cloutier, S., Sharpe, A. G., Wong, G. K., Wang, J., and Deyholos, M. K. (2012) The genome of flax (*Linum usitatissimum*) assembled *de novo* from short shotgun sequence reads. *Plant J.* **72,** 461–473
18. Abascal, F., Zardoya, R., and Telford, M. J. (2010) TranslatorX: multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Res.* **38,** W7–W13
19. Suyama, M., Torrents, D., and Bork, P. (2006) PAL2NAL: Robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* **34,** W609-W612
20. Nei, M., and Kumar, S. (2000) *Molecular Evolution and Phylogenetics* (electronic resource), Oxford University Press, New York
21. Gibson, D. G. (2011) Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol.* **498,** 349–361
22. Goldstein, A. L., and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15,** 1541–1553
23. Sandager, L., Gustavsson, M. H., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., and Stymne, S. (2002) Storage lipid synthesis is non-essential in yeast. *J. Biol. Chem.* **277,** 6478–6482
24. Chen, Q., Kazachkov, M., Zheng, Z., and Zou, J. (2007) The yeast acylglycerol acyltransferase LCA1 is a key component of Lands cycle for phosphatidylcholine turnover. *FEBS Lett.* **581,** 5511–5516
25. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **21,** 3329–3330
26. Gietz, R. D., and Schiestl, R. H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2,** 31–34
27. Hegemann, J. H., and Heick, S. B. (2011) Delete and repeat: A comprehensive toolkit for sequential gene knockout in the budding yeast *Saccharomyces cerevisiae*. *Methods Mol. Biol.* **765,** 189–206
28. Covello, P. S., and Reed, D. W. (1996) Functional expression of the extraplastidial *Arabidopsis thaliana* oleate desaturase gene (FAD2) in *Saccharomyces cerevisiae*. *Plant Physiol.* **111,** 223–226
29. Dyer, J. M., Chapital, D. C., Cary, J. W., and Pepperman, A. B. (2001) Chilling-sensitive, post-transcriptional regulation of a plant fatty acid desaturase expressed in yeast. *Biochem. Biophys. Res. Commun.* **282,** 1019–1025
30. Tang, G. Q., Novitzky, W. P., Carol Griffin, H., Huber, S. C., and Dewey, R. E. (2005) Oleate desaturase enzymes of soybean: Evidence of regulation through differential stability and phosphorylation. *Plant J.* **44,** 433–446
31. Byers, S. D., Laroche, A., Smith, K. C., and Weselake, R. J. (1999) Factors enhancing diacylglycerol acyltransferase activity in microsomes from cell-suspension cultures of oilseed rape. *Lipids* **34,** 1143–1149
32. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917

33. Siloto, R. M., Truksa, M., He, X., McKeon, T., and Weselake, R. J. (2009) Simple methods to detect triacylglycerol biosynthesis in a yeast-based recombinant system. *Lipids* **44,** 963–973
34. Sørensen, B. M., Furukawa-Stoffer, T. L., Marshall, K. S., Page, E. K., Mir, Z., Forster, R. J., and Weselake, R. J. (2005) Storage lipid accumulation and acyltransferase action in developing flaxseed. *Lipids* **40,** 1043–1049
35. Weiss, S. B., Kennedy, E. P., and Kiyasu, J. Y. (1960) The enzymatic synthesis of triglycerides. *J. Biol. Chem.* **235,** 40–44
36. Wickramarathna, A. D., Siloto, R. M. P., Mietkiewska, E., Singer, S. D., Pan, X., and Weselake, R. J. (2015) Heterologous expression of flax phosphotidylcholine:diacylglycerol cholinephosphotransferases (*PDCTs*) increases polyunsaturated fatty acid content in yeast and *Arabidopsis* seeds. *BMC Biotechnol.,* in press
37. Wang, L., Shen, W., Kazachkov, M., Chen, G., Chen, Q., Carlsson, A. S., Stymne, S., Weselake, R. J., and Zou, J. (2012) Metabolic interactions between the Lands cycle and the Kennedy pathway of glycerolipid synthesis in *Arabidopsis* developing seeds. *Plant Cell* **24,** 4652–4669
38. Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I., and Browse, J. (2008) Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. *Plant Biotechnol. J.* **6,** 819–831

# *In Vivo* and *in Vitro* Evidence for Biochemical Coupling of Reactions Catalyzed by Lysophosphatidylcholine Acyltransferase and Diacylglycerol Acyltransferase

Xue Pan, Guanqun Chen, Michael Kazachkov, Michael S. Greer, Kristian Mark P. Caldo, Jitao Zou and Randall J. Weselake

*J. Biol. Chem. 2015, 290:18068-18078.*
*doi: 10.1074/jbc.M115.654798 originally published online June 8, 2015*