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Brief Communication

Acyl-CoA:lysophosphatidylcholine acyltransferase from the unicellular diatom *Phaeodactylum tricornutum* (PtLPCAT1) is involved in triacylglycerol and galactoglycerolipid synthesis and enhances eicosapentaenoic acid accumulation in recombinant oleaginous yeast

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Dietary supplementation of omega-3 very long-chain polyunsaturated fatty acids (VLC-PUFAs), particularly eicosapentaenoic acid (EPA, C20 : 5 $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, C22 : 6 $\Delta^{4,7,10,13,16,19}$), is well known to have diverse health benefits. In addition to their major source of marine fish, metabolic engineering of oilseed crops could provide a sustainable alternative source for these pharmaceutically important FAs. The reconstruction of VLC-PUFAs biosynthetic pathway in plants involves the assembly of multiple desaturases and elongases from different organisms. However, when phospholipid-linked desaturases and acyl-CoA-dependent elongases were co-expressed, the so-called substrate dichotomy was predicted to generate a metabolic bottleneck (i.e. low efficiency in the channelling of lipid-linked desaturation intermediates into the acyl-CoA pool) for the accumulation of high levels of VLC-PUFAs in transgenic plants (Abbadì *et al.*, 2004).

Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT, E.C. 2.3.1.23) has previously been thought to be the possible candidate enzyme regulating this bottleneck. The diatom *Phaeodactylum tricornutum* produces EPA up to ~30% of total fatty acids. Eicosapentaenoic acid biosynthesis proceeds predominantly via the so-called $\Delta 6$ -pathway in the endoplasmic reticulum (ER), which prompts us to identify LPCAT from this microalga. Recently, the biochemical properties of one *P. tricornutum* LPCAT

(PtLPCAT1) were reported and it can esterify a 18 : 3-, 18 : 4- or 20 : 4-CoA (n-3) moiety to lysoPC *in vitro*, the later acyl being available for further desaturation to synthesize EPA, contrasting with plant LPCATs (Polońska *et al.*, 2021). However, its role in lipid synthesis and the potential to enhance VLC-PUFAs production remain unknown.

Here, we found that PtLPCAT1 fused with green fluorescence protein was localized at the chloroplast ER membrane (Figure 1a). To disrupt *PtLPCAT1*, we constructed a multiplexed CRISPR/Cas9 plasmid (Figure 1b) and transformed into *P. tricornutum*. We obtained at least 3 independent mutant lines harbouring mutations at the intended target site based on PCR and DNA sequencing (Figure 1c,d). To analyse lipid-associated phenotypes, *P. tricornutum* wild-type (WT) and the *PtLPCAT1* knockout mutants were grown in nutrient-replete and phosphorus-depleted conditions. During 10 days of growth, cell density and neutral lipid content in *PtLPCAT1* were significantly decreased compared with WT (Figure 1e,f). The mutants also accumulated less triacylglycerols (TAGs) than WT (Figure 1g). Although there was no difference in cell densities between WT and *PtLPCAT1* grown in phosphorus-depleted condition for 7 days, the mutants showed a significant decrease in Nile red fluorescence and TAG accumulation (Figure 1h–j), which suggesting that disruption of *PtLPCAT1* led to compromised cell growth and impaired TAG synthesis. Additionally, EPA was significantly decreased in *PtLPCAT1* at Day 9 ($P < 0.05$), and the levels of C16 : 0 and C16 : 1 were reduced in *PtLPCAT1* at both Day 6 and Day 9 (Figure 1k).

Glycerolipidomic analysis revealed that inactivation of *PtLPCAT1* caused a significant decrease in the levels of TAGs (30%, $P < 0.001$) and monogalactosyldiacylglycerol (MGDG, 20%, $P < 0.05$) at 9 day, and an increase in the amounts of phosphatidylcholine (PC) (50%, $P < 0.01$) and phosphatidylethanolamine (PE) (50%, $P < 0.01$) compared with WT (Figure 1l). Quantitative analysis of molecular species in each lipid class revealed that molecular

composition of the major chloroplast galactoglycerolipids was influenced in *PtLPCAT1*. The most abundant 20 : 5/16 : 3 species in MGDG was decreased from 30% in WT to 15% in the mutants at 6 d ($P < 0.0001$). In addition, almost all other molecular species harbouring a 20 : 5 at the *sn*-1 position also showed a significant decrease in the mutants correlated with a dramatic

increase in the 16 : 0/16 : 0 and 16 : 1/16 : 0 species ($P < 0.0001$; Figure 1m). Similarly, a significant decrease in C16-FA was observed in 20 : 5-containing molecular species of digalactosyl-diacylglycerol (DGDG) in the *PtLPCAT1* mutants (Figure 1n). For extra-plastidic glycerolipids, disruption of *PtLPCAT1* caused a decrease in the levels of 18 : 2/18 : 1 and 18 : 2/18 : 2 in PC and

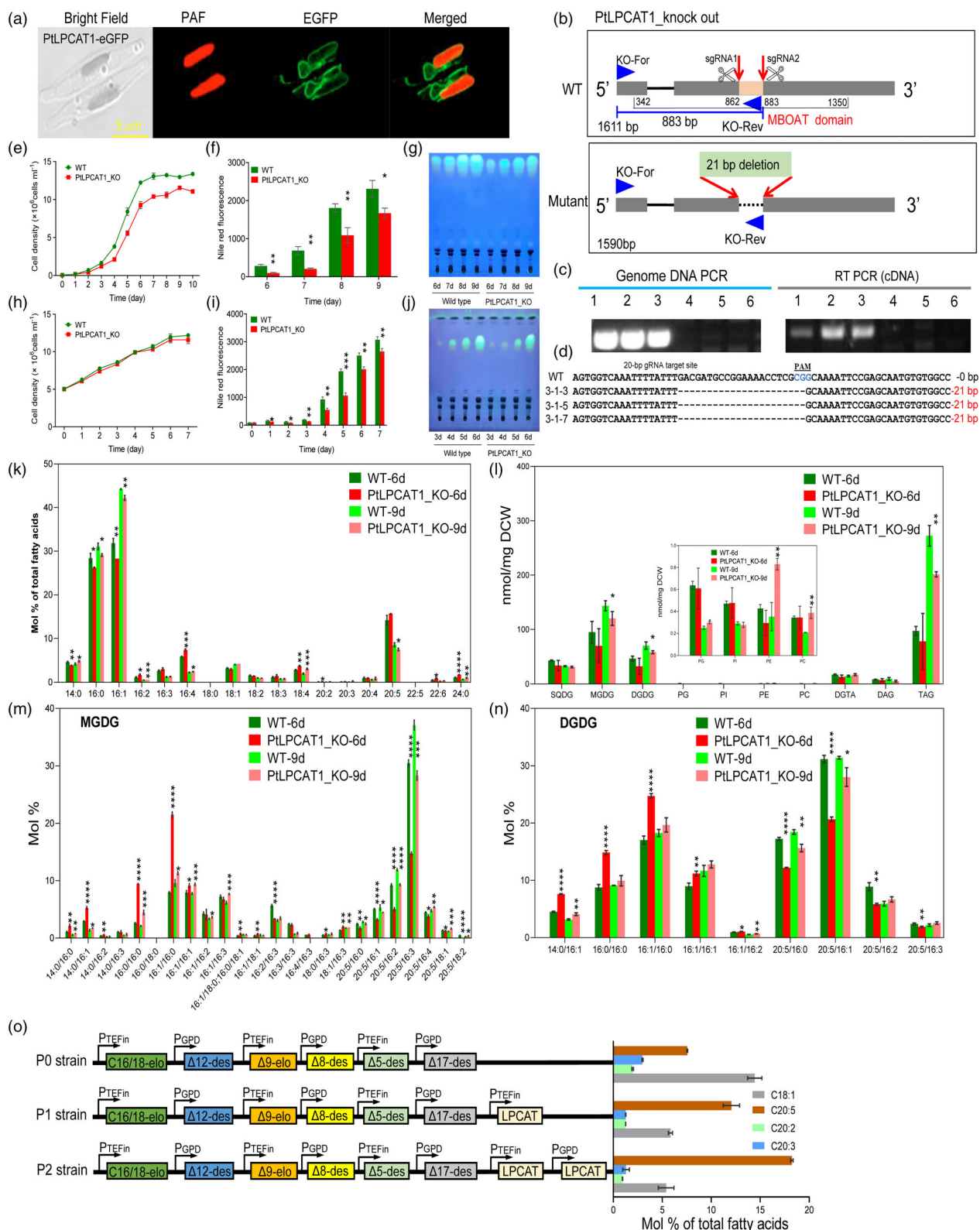


Figure 1 Role of PtLPCAT1 in glycerolipid synthesis in *P. tricornutum* and the potential to enhance EPA accumulation in recombinant yeast *Y. lipolytica*. (a) Subcellular localization of PtLPCAT1. PAF, plastid autofluorescence. (b) Schematic illustration of CRISPR/Cas9 for disruption of *PtLPCAT1*. (c) PCR amplification was used to detect a fragmental deletion within *PtLPCAT1*. (d) DNA sequencing for representative mutation aligned with wild type. Cell growth (e), Nile red fluorescence intensities (f) and thin-layer chromatogram (TLC) of total lipids (g) were analysed for wild-type (WT) and *PtLPCAT1* grown in nutrient-replete condition. Cell growth (h), Nile red fluorescence intensities (i) and TLC (j) were analysed for WT and *PtLPCAT1* grown in phosphorus-deprived condition for 7 days. Data are the average of three biological replicates with error bars indicating standard deviations ($n = 3$). Statistical analysis using paired-sample Student's *t* test was performed: * $P < 0.05$; ** $P < 0.01$. Fatty acid profiles (k) and the amount of each glycerolipid class (l) of WT and *PtLPCAT1* grown in nutrient-replete medium were shown. The proportion of each molecular species in MGDG (m) and DGDG (n) was compared between WT and *PtLPCAT1* grown in nutrient-replete medium. *** $P < 0.001$; **** $P < 0.0001$. (o) Overexpression of PtLPCAT1 in recombinant EPA-producing *Y. lipolytica* strain could significantly enhance the percentage of EPA in total fatty acids.

20 : 5/20 : 5 in diacylglyceryl-hydroxymethyl-*N,N,N*-trimethyl- β -alanine (DGTA) (Table S1). Both 18 : 3 and 18 : 4 accumulated in PC and DGTA, suggesting that PtLPCAT1 was not involved in their transfer and was more specific for 20 : 4 *in vivo*. Phosphatidylcholine and DGTA are the putative extra-plastidic platforms for FA desaturation, and their turnover may provide substrates for chloroplast galactolipid synthesis. In PE, the *PtLPCAT1* mutants also showed a significant decrease in the level of 20 : 5/20 : 5 in the exponential phase when compared to WT. The changes in molecular composition of PC, DGTA and chloroplast galactolipids are consistent with a role of PtLPCAT1 in acyl editing of PC and possibly DGTA. The most significant alterations of TAGs were observed in the reduction of C20 : 5-containing species, balanced by an increase in C16-containing species, at both Day 6 and Day 9 (Figure S1, Table S1). These changes demonstrate that disruption of *PtLPCAT1* caused a decrease in the C20 : 5-containing molecular species in galactoglycerolipids and TAGs.

To evaluate the potential of PtLPCAT1 to enhance VLC-PUFA production, we reconstructed EPA biosynthetic pathway in the nonconventional oleaginous yeast *Yarrowia lipolytica*, which is an attractive host for the production of oils and FA-derived compounds. We first constructed an EPA-producing starting strain (YALI_P0) through integration of multiple gene expression cassettes into the PO1f strain. The FA profile of *Y. lipolytica* PO1f strain revealed that it can synthesize linoleic acid (C18 : 2 n-6) and oleic acid (C18 : 1 n-9) as the major FAs. To produce EPA from linoleic acid through an alternative pathway relying on the initiating $\Delta 9$ -elongation step in the acyl-CoA pool followed by phospholipid-linked desaturations, we introduced a $\Delta 9$ -elongase, $\Delta 8$ -desaturase, $\Delta 5$ -desaturase from *Euglena gracilis* and a $\Delta 17$ -desaturase from *Pythium aphanidermatum*, all of which were codon-optimized for expression in *Y. lipolytica*. Insertion of these DNA fragments into the *Y. lipolytica* genome occurred by homologous recombination, and this led to the accumulation of EPA up to 7% of total FAs in the YALI_P0 strain. We then used pCfB-PtLPCAT1 and pCfB-2 \times PtLPCAT1, which harbour one copy and two copies of a PtLPCAT1 gene, respectively, to transform strain YALI_P0. The resulting strains YALI_P1 and YALI_P2 produced EPA at 12.06% and 18.23% of total FAs (Figure 1o), respectively, which indicated that overexpression of PtLPCAT1 could significantly enhance EPA production in recombinant *Y. lipolytica* and the final percentage of EPA in total FAs was associated with the copy number of PtLPCAT1 gene. Moreover, compared with YALI_P0 strain, we observed a marked reduction in the amount of C20-FA intermediates such as $\Delta 9$ -elongation products (C20 : 2 $\Delta^{11,14}$ and C20 : 3 $\Delta^{11,14,17}$) in PtLPCAT1-overexpressed strains YALI_P1 and YALI_P2, indicating that introduction of additional PtLPCAT1 activity may accelerate, at least in part, the exchange of $\Delta 9$ -elongation product (C20 : 3 $\Delta^{11,14,17}$) between

phospholipids and the acyl-CoA pool and thereby push the intermediates towards the synthesis of the end product, EPA.

In summary, our work reveals the important role of PtLPCAT1 in lipid synthesis in *P. tricornutum* and demonstrates the enhanced EPA accumulation in the yeast *Y. lipolytica* through overexpression of PtLPCAT1, which may also have the potential to enhance the accumulation of VLC-PUFAs in transgenic oilseed plants.

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Conflicts of interest

A related patent had been submitted to the State Intellectual Property Office of China.

Author contributions

L.Y., X.H. and D.Z. performed the experiments. J.J., E.M., A.A. and A.B. analysed the data. Y.G. designed the study and wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Profiling of molecular species of TAGs.

Table S1 Lipidomic dataset.