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Multiplex CRISPR/Cas9 editing of the long-chain acyl-CoA synthetase family in the diatom *Phaeodactylum tricornutum* reveals that mitochondrial ptACSL3 is involved in the synthesis of storage lipids

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1 **Multiplex CRISPR/Cas9 editing of the long-chain acyl-CoA**
2 **synthetase family in the diatom *Phaeodactylum tricornutum* reveals**
3 **that mitochondrial ptACSL3 is involved in the synthesis of storage**
4 **lipids**

5

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37 Summary

38 ● Long-chain acyl-CoA synthetases (LACSs) play diverse and fundamentally important roles in
39 lipid metabolism. While their functions have been well-established in bacteria, yeast and plants,
40 the mechanisms by which LACS isozymes regulate lipid metabolism in unicellular oil-producing
41 microalgae, including the diatom *Phaeodactylum tricornutum*, remain largely unknown.

42 ● In *P. tricornutum*, a family of five genes (*ptACSL1~5*) encodes LACS activities. We
43 generated single *lacs* knockout/knockdown mutants using multiplexed CRISPR/Cas9 method, and
44 determined their substrate specificities towards different fatty acids (FAs) and subcellular
45 localizations.

46 ● *ptACSL3* is localized in the mitochondria and its disruption led to compromised growth and
47 reduced triacylglycerol (TAG) content when cells were bubbled with air. The *ptACSL3* mutants
48 showed altered FA profiles in two galactoglycerolipids and phosphatidylcholine (PC) with
49 significantly reduced distribution of 16:0 and 16:1. *ptACSL5* is localized in the peroxisome and its
50 knockdown resulted in reduced growth rate and altered molecular species of PC and TAG,
51 indicating a role in controlling the composition of acyl-CoAs for lipid synthesis.

52 ● Our work demonstrates the potential of generating gene knockout mutants with the mutation
53 of large fragment deletion using multiplexed CRISPR/Cas9 and provides insight into the functions
54 of LACS isozymes in lipid metabolism in the oleaginous microalgae.

55

56 **Key words:** Fatty acid, Long-chain acyl-CoA synthetase, Multiplexed CRISPR/Cas9,

57 *Phaeodactylum tricornutum*, Triacylglycerol.

58

59 **Introduction**

60 Fatty acids (FAs), a highly diverse class of molecules, have variable chain length and number
61 of double bonds and are building blocks for all membrane lipids and storage lipids in the cells.
62 They are essential molecules in almost all organisms. Different from that in animals and fungi,
63 which produce FAs primarily in the cytosol for lipid biosynthesis, in plants *de novo* synthesis of
64 FAs takes place in the plastid via a repeated cycle of reactions with fatty acyl intermediates
65 covalently attached to acyl carrier protein (ACP), providing 16:0-, 18:0- and 18:1-ACP as the final
66 products (Ohlrogge and Browse, 1995). These fatty acyl-ACP thioesters can serve as substrates to
67 plastid-localized acyltransferases and feed directly into the “prokaryotic” lipid synthesis pathway
68 to produce phosphatidylglycerol (PG) and other thylakoid lipids such as
69 sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG), and
70 digalactosyldiacylglycerol (DGDG) in the plastid (Heinz and Roughan 1983; Jessen et al. 2015).
71 Alternatively, the current model proposes that a part of acyl-ACPs are hydrolyzed in the plastid
72 stroma by the action of acyl-ACP thioesterase to release free FAs that are targeted for export from
73 the plastid. Once activated into fatty acyl-CoAs, these acyl groups can enter the eukaryotic
74 pathway in the endoplasmic reticulum (ER) for incorporation into extraplastid membrane lipids
75 and storage lipids. In addition, plastid-derived FAs are also substrates for eukaryotic pathway to
76 synthesize important lipid precursors, like phosphatidic acid (PA) and diacylglycerol (DAG),
77 which are imported back to the plastid for thylakoid lipid biosynthesis. This two-pathway
78 hypothesis for thylakoid lipid biosynthesis was originally postulated by Roughan and Slack
79 (Roughan et al., 1980; Roughan and Slack, 1982), supported by specific acyl compositions of lipid
80 molecular species derived from the plastid and ER pathways (Heinz and Roughan 1983), and later
81 corroborated by genetic and biochemical analysis of several mutants in the model plant
82 *Arabidopsis thaliana* (Kunst et al. 1988; Wallis and Browse 2002; Xu et al. 2008). In contrast to
83 plants that contain the primary plastids with a two-membrane envelope, evidence is lacking to
84 support such a similar pathway for phospholipid trafficking from the ER to chloroplasts to form
85 galactolipids in photosynthetically active stramenopiles with a secondary plastid surrounded by
86 four limiting membranes, such as the eustigmatophyte *Nannochloropsis* sp. and the diatom *P.*
87 *tricornutum*. In these photosynthetic eukaryotes, MGDG and DGDG are characterized by a high
88 content of 20:5, which is synthesized in the ER from the initial substrate of plastid-exported 16:0.

89 After synthesis in the ER, 20:5 needs to be imported into the plastid through the unknown “omega
90 pathway” (Petroustos et al. 2014; Dolch et al. 2017).

91 In plants, before becoming accessible to the “eukaryotic” lipid synthesis pathway, free FAs
92 need to be activated by esterification with CoA to form acyl-CoA thioesters as they are chemically
93 inert. The activation of free FAs to form fatty acyl-CoAs is catalyzed by long-chain acyl-CoA
94 synthetases (LACSs, EC 6.2.1.3). This reaction occurs in two steps. First, the free FA reacts with
95 ATP to generate an intermediate fatty acyl-AMP (acyl adenylate) with the release of
96 pyrophosphate. In the second step, the enzyme bound acyl-AMP reacts with CoA to form acyl-
97 CoA, with the release of AMP (Groot et al., 1976). In photosynthetic stramenopiles, LACS
98 activity is likely to be not only required for activation of free FAs (16:0 and 16:1) exported from
99 the plastid, but also involved in the downstream 16:0-to-20:5 route in which it provides activated
100 FAs for FA elongases and acyltransferases.

101 LACS activities have been localized in various subcellular compartments including the
102 chloroplast, cytoplasm, ER, peroxisome, mitochondria and oil bodies. The physiological function
103 of LACS in cellular homeostasis, particularly in lipid metabolism, has been studied in detail in
104 bacteria (Black and DiRusso 1994; Black et al. 1997), yeast (Færgeman et al. 2001; Zou et al.
105 2003) and plants (Lü et al. 2009; Weng et al. 2010; Jessen et al. 2011, 2015; Zhao et al. 2010,
106 2019). Like higher plants, plastids of photosynthetic microalgae are also the principle site for *de*
107 *novo* FA synthesis while further desaturation and chain elongation occur in the ER and fatty acyl
108 catabolism takes place in the mitochondria and/or peroxisome via β -oxidation. We previously
109 reported the identification of five LACS genes in the diatom *Phaeodactylum tricornutum* that can
110 synthesize and accumulate large amounts of TAGs at stationary phase of growth and under stress
111 conditions, particularly nitrogen starvation (Guo et al. 2014). Although the LACS isozymes have
112 been identified in *Phaeodactylum*, the functional role of individual LACS in lipid metabolism,
113 especially their contribution to storage lipid biosynthesis, remains largely unknown. In the past ten
114 years, development of genome-editing techniques, such as the mutagenesis with transcription
115 activator-like effector nucleases (TALENs) and the CRISPR/Cas9 method (Weyman et al. 2015;
116 Daboussi et al. 2014; Serif et al. 2018; Moosburner et al. 2020), has allowed us to generate
117 targeted and stable modification of the diatom genome to address the functions of key genes.
118 Considering acyl-CoA thioesters as key building blocks required for acyl-CoA-dependent TAG

119 biosynthesis, we here used multiplexed CRISPR/Cas9 method to generate *lacs* single mutants and
120 to assess the contribution of each ptLACS isozyme to lipid biosynthesis. In addition, we
121 determined their subcellular localizations, protein expression patterns and enzyme activities. Our
122 results advance the understanding on the functions of LACS isozymes in lipid metabolism in oil-
123 accumulating unicellular microalgae.

124

125 **Materials and methods**

126 **Strains and growth conditions**

127 *P. tricornutum* strain Pt1 WT and derived mutants were grown in artificial seawater (ASW)
128 media with F/2 nutrients (Guillard 1975) at 22°C under white fluorescent lights (50 $\mu\text{mol photons}$
129 $\text{m}^{-2} \text{s}^{-1}$) with a 12h:12 h light-dark cycle, and additional supplementation of 100 mg L^{-1} Zeocin was
130 included for mutants. For time-course growth experiments in static culture, 200-mL algal cultures
131 of were grown in batch mode for 12 d with an initial addition of inoculum to give a starting cell
132 density of around 5×10^6 cells mL^{-1} . For growth experiments with aeration, duplicate 300-mL
133 cultures were bubbled for 9 d with air supply sterilized by filtration through 0.22 μm Millipore
134 filters. Samples were taken daily for cell counting and measurement of Nile Red fluorescence and
135 nitrate concentration. For the immunoblot experiment, algal cells were grown in N-replete F/2
136 medium to the exponential phase and then transferred to N-free medium for additional 72 h, or to
137 N-replete medium bubbled with air, 1%, 4% and 10% CO_2 enriched air for additional 6 h.

138 **Plasmid construction**

139 Plasmids for generation of *Phaeodactylum ptACSL* mutants using CRISPR/Cas9 method
140 were constructed as described by Moosburner et al. (2020). To construct eGFP fusion plasmids,
141 full-length ptACSL sequences were cloned into the multiple cloning sites (MCSs) of the
142 expression vector pPha-CG/ pPha-NG. For self-assembling split GFP constructs, we used the
143 pPha-Dual vector that harbors two MCSs for gene cloning. Details are described in supporting
144 information [Methods S1](#). All the plasmids and primer sequences used in this study were listed in
145 [Table S1](#) and [S2](#).

146 **Generation of ptACSL1-5 knock out/down lines**

147 For editing of each *ptACSL* gene, six sgRNAs were designed in the coding region, namely gRNA1
148 to gRNA6. Every three sites were designed to be assembled in a single vector carrying Cas9 and

149 zeocin resistance cassette via Golden Gate cloning. Transformation of *Phaeodactylum* via
150 bacterial-mediated conjugation and screening were carried out as described previously
151 (Moosburner et al. 2020). For genotypic characterization, algal cell lysates served as the PCR
152 templates and primers listed in Table S2 were used to amplify the various loci of interest. Cas9-
153 induced mutation for each *ptACSL* gene was analyzed by direct sequencing of PCR products
154 containing the targeted sites. The Inference of CRISPR Edits tool (ICE, Synthego,
155 <https://ice.synthego.com>) was used to genotype *Phaeodactylum* mutant lines. It offers the indel
156 (insertion or deletion) type and the overall indel percentage (editing efficiency) by decoding of all
157 the sequencing chromatograms with monoallelic mutation, homozygous and heterozygous
158 biallelic mutations, which result in superimposed sequencing chromatograms.

159 **In vitro LACS enzyme assay**

160 Recombinant *Phaeodactylum* ptACSL proteins were produced from *E. coli* BL21 (DE3) pLysS and
161 purified with Ni-NTA affinity column (Novagen). LACS enzyme activity was measured
162 spectrophotometrically using an enzyme-coupled assay (Horswill and Escalante-Semerena 2002;
163 [Methods S1](#)). Determination of kinetic constants of recombinant ptACSLs was described in [Method](#)
164 [S1](#).

165 **Transformation of *Phaeodactylum tricornutum***

166 All the plasmids of GFP fusions were transformed into *Phaeodactylum* by microparticle
167 bombardment using Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad
168 Laboratories, Hercules, CA) as previously described ([Zaslavskaja et al. 2000](#)). Bacterial-mediated
169 conjugation was used to introduce Cas9-ShBle:sgRNA episome into *Phaeodactylum* as described
170 by Karas et al. (2015). Resistant colonies were selected on F/2 media supplemented with 100 μg
171 mL^{-1} zeocin.

172 **Immunoblotting**

173 Antibodies for ptACSL1 and ptACSL4 were generated as described previously ([Chen et al. 2018](#)).
174 The polyclonal antibodies that specifically recognize ptACSL2, ptACSL3 and ptACSL5 were raised
175 by immunizing rabbits with purified full-length recombinant proteins. The expression of ptACSL1-
176 5 was examined by immunoblotting as described in [Methods S1](#).

177 **Lipid extraction and analysis**

178 Glycerolipids from *Phaeodactylum* WT and the *ptACSL* mutants were extracted as described by

179 Abida et al. (2015). The *sn*-position of FAs on individual glycerolipids and quantification of each
180 molecular species were analyzed by preferential loss using MS and MS2 analysis and liquid
181 chromatography-MS/MS, respectively, as previously described (Jouhet et al. 2017). For detailed
182 analysis of the change of TAG molecular species, TAGs were isolated by TLC from total lipids of
183 WT and the *ptACSL3* mutants grown at static (day 11) and air-bubbled (day 7) conditions, and LC-
184 MS method was used to quantify each molecular species of TAGs as described by Xie et al. (2020)
185 (Methods S1).

186 RNA-seq analysis

187 WT and the *ptACSL3* mutants were grown in static culture for 6 d and then transferred to the cultures
188 bubbled with filtered air for additional 4 d. Cells were harvested at 2 d, 3 d, and 4 d for RNA-seq
189 and samples under static culture for 6 d were used as control (0 d). Three biological replicates of
190 WT and the *ptACSL3* mutant and of each condition were processed for total RNA isolation using
191 TRIzol reagent (Invitrogen, USA), and RNA sequencing were performed by the Illumina NovaSeq
192 6000 platform. RNA-seq reads were aligned to the Ensemble reference genome and gene model
193 annotation files. The DESeq2 R package (1.20.0) was used to detect differential expressed genes
194 (DEGs) between the WT and mutants. The DEGs were subjected to gene ontology (GO) enrichment
195 analysis with a clusterProfiler R package (Methods S1).

196

197 Results

198 Substrate specificities of ptACSL1–5 determined by *in vitro* enzyme assay

199 The purified recombinant ptACSL proteins served as enzyme sources in LACS activity assays,
200 using different free FAs as a substrate. The tested substrates are endogenous FAs and some are
201 present in a high proportion in acyl lipids of *Phaeodactylum*. We used an enzyme-coupled method
202 for measurement of LACS activity (Horswill and Escalante-Semerena 2002). Substrate specificities
203 and catalytic efficiencies of ptACSL1–5-catalyzed reactions were determined by varying
204 concentrations of free FAs with fixed saturating concentrations of ATP and CoA. The plots of initial
205 velocities versus free FA concentrations were fitted to the Michaelis-Menten equation to determine
206 the K_m , k_{cat} , and k_{cat}/K_m values. As shown in Table 1, K_m values for all five ptACSLs were in the
207 range of 27–451 μM , and all five ptACSL enzymes exhibited LACS activity for oleic acid (18:1n-
208 9) with k_{cat}/K_m values ranging from 1851 to 5912 $\text{M}^{-1} \text{s}^{-1}$. When supplied with different FA substrates,
209 ptACSL1 proved to be the most active and have the highest catalytic efficiency (k_{cat}/K_m , the constant
210 that better measures the specificity for the substrate), with EPA, with a value exceeding by 2 orders

211 of magnitude that measured in presence of 16:0. Similarly, ptACSL4 also displayed a preference for
212 EPA, with a k_{cat}/K_m value 5- and 184-fold higher than those obtained with the two other FAs tested.
213 Additionally, of the five LACS enzymes, ptACSL5 is only LACS showing no any activity for EPA.
214 The K_m values determined for ptACSL5, which reflect the binding affinity of an enzyme for a
215 substrate, indicate that ptACSL5 had the greatest affinity for 18:0 with K_m value of 45 μ M. These
216 data show that recombinant ptACSL proteins exhibit a difference in specificities towards free FAs,
217 and ptACSL1 and ptACSL4 have the highest catalytic efficiency for EPA, the most abundant very
218 long-chain polyunsaturated FA (VLC-PUFA) detected in *Phaeodactylum*.

219 **Subcellular localization of LACS isozymes in *Phaeodactylum***

220 ptACSL1 was previously reported to be localized in the plastid (Chen et al. 2018). To study its
221 precise membrane location, we used the GFP self-assembling system (Cabantous et al., 2005) to
222 determine the localization and orientation of ptACSL1. In this GFP reporter system, the last beta
223 strand of GFP (GFP_S11) was attached to the C-terminus of ptACSL1 and the beta strands 1-10
224 (GFP_S1-10) were C-terminally fused either to a cER (chloroplast endoplasmic reticulum) marker
225 to be expressed in the ER lumen or expressed as a cytosolically localized large GFP_S1-10 fragment.
226 These constructs were transformed into *Phaeodactylum*, and a cER-characteristic GFP fluorescence
227 could be observed only when a fusion protein consisting of full-length sequence of ptACSL1 and
228 GFP_S11 was simultaneously expressed with the large GFP_S1-10 fragment. In contrast,
229 expression of the same ptACSL1 fusion protein with a cER-targeted PDI-GFP_S1-10 (PDI) fusion
230 construct resulted in the absence of detectable fluorescence signal (Fig.1). This indicates a
231 localization of ptACSL1 in the cER or cER membrane (outermost membrane of the complex plastid)
232 with a C-terminal orientation towards the cytoplasm. The hydrophobicity plot predicted a short
233 transmembrane region at the N-terminus of ptACSL1, it is thus possibly localized in the cER
234 membrane with the C-terminus facing the cytoplasm.

235 The attachment of ptACSL2 to GFP (ptACSL2:eGFP) caused GFP fluorescence to be located
236 in the cytoplasm. In the transformants harboring ptACSL4:GFP fusion construct, GFP signal was
237 also mainly dispersed throughout the cytoplasm. Fusion of ptACSL3 with GFP led to the
238 mitochondria-characteristic fluorescence, indicating its location in the mitochondria that are in close
239 proximity to the complex plastid. A peroxisomal targeting signal (PTS) type 1 (SKL), which is
240 necessary for targeting and import of cytosolically expressed proteins into peroxisomes, was found
241 to be located at the extreme C-terminus of the ptACSL5 isoform. For *in vivo* localization study we

242 fused eGFP upstream of full-length sequence of ptACSL5 to ensure the PTS remained accessible,
243 and this eGFP:ptACSL5 fusion construct resulted in a punctate GFP fluorescence pattern (Fig.1),
244 confirming its peroxisomal localization in transfected *Phaeodactylum* cells.

245

246 **Immunoblot analyses of ptACSL accumulation in response to nitrogen deprivation and** 247 **elevated CO₂**

248 Nitrogen-starved *Phaeodactylum* cells are capable of accumulating large amounts of TAGs,
249 the major storage compounds for FAs. Lipid accumulation also increased in *Phaeodactylum* cells
250 grown in seawater in response to elevated atmospheric CO₂ concentration (Jensen et al. 2019). We
251 thus studied the expression of LACSs in response to nitrogen deprivation and increased CO₂ supply.
252 Using ptACSL-specific antibody (Fig.S1), we detected the protein levels by Western blot in
253 *Phaeodactylum* cells grown in F/2 media with nitrogen deprivation and supplied with different
254 concentrations of CO₂. As shown in Fig.2a, the expression of ptACSL1–4 proteins was regulated
255 by nitrogen deprivation while ptACSL5 showed a constant expression, suggesting differential
256 expressions of these LACS isozymes upon nitrogen deprivation at protein levels. The expression
257 of ptACSL1 and ptACSL3 was induced by nitrogen deprivation and their abundances peaked at 72
258 h. By contrast, the level of ptACSL2 was very high under nitrogen-replete condition but it declined
259 during the time course of nitrogen deprivation. When cells were grown at atmospheric CO₂ and at
260 high CO₂ (1%, 4% and 10%), the expression of ptACSL1, 2 and 4 was slightly suppressed at high
261 CO₂ compared to that at atmospheric CO₂. Notably, the expression of ptACSL3 was upregulated at
262 elevated CO₂ and reached the highest level at 4% CO₂ concentration followed by a decline at 10%
263 CO₂ concentration (Fig.2b).

264 **Multiplex CRISPR/Cas9 system induced mutation of single ptACSL gene**

265 We used multiplex CRISPR/Cas9 genome editing system to generate single knockout (KO)
266 mutants for each *ptACSL* gene (Moosburner et al. 2020). For creating the single gene mutants for
267 all five ptACSL genes, we prepared ten Cas9-sgRNA episome-based constructs, each carrying three
268 sgRNA expression cassettes to target three different sites within a single *ptACSL* gene (Fig.3a). The
269 final vectors expressing Cas9 and the sgRNA assay were transformed into *Phaeodactylum*, and PCR
270 followed by Sanger sequencing was conducted to detect targeted mutations. Detailed analysis of
271 total 246 sequenced sites from all five ptACSL genes showed that most colonies have a mixed

272 genotype, which consists of wild-type (WT) sequence, nucleotide deletion and a very low
273 percentage of nucleotide insertion. We compared the editing efficiencies of target mutations induced
274 by different numbers of gRNAs, and found that mutation frequency of nucleotide insertion induced
275 by double gRNAs was two-fold higher than that induced by a single gRNA. In double gRNAs-
276 induced mutations, the frequencies of deletions with 1-10 bp in size was slightly higher than that of
277 single gRNA-induced mutations while the frequencies of deletions with 10-30 bp in size were
278 similar (Fig.3b). Notably, we also detected homozygous biallelic mutations with large fragment
279 deletions (158-230 bp) between two cleavage sites in some colonies. Homozygous biallelic
280 mutations occurred in a varied frequency among different *ptACSL* genes, with the highest
281 mutagenesis frequency of 30% in *ptACSL4* and the lowest frequency of 2.5% in *ptACSL1* (Fig.3c).
282 We propagated these *Phaeodactylum* mutant lines in liquid media and then plated dilutions to obtain
283 single colonies. Further sequencing was conducted to detect the targeted mutations, and it showed
284 that the percentage of homozygous biallelic mutations was greatly increased. Finally, we confirmed
285 the absence of wild-type gene using PCR with genomic DNA prepared from these sequenced single
286 colonies. For this purpose, we designed a pair of primers with one being located in the region of
287 fragmental deletion (between two active cleavage sites) while the other outside it and corresponding
288 to the non-editing region (Figs 4a, 4b). For instance, genomic PCR showed that the expected DNA
289 bands could be observed in WT/control colonies, while there was no DNA band in the *ptACSL1* or
290 *ptACSL3* KO mutants (Figs 4c, 4d). Mutation of long fragment deletions was also verified using
291 reverse transcription PCR (RT-PCR), indicating the complete lack of detectable expression of
292 *ptACSL* genes (Figs 4e, 4f). Using this method, we obtained the single KO mutants for *ptACSL1*,
293 *ptACSL2*, *ptACSL3*, and *ptACSL4*, and knockdown mutants for *ptACSL5* (Figs 4, S2). Therefore,
294 creating long deletions with multiple gRNAs should be a more efficient approach to achieve targeted
295 mutagenesis, and a rapid and low-cost PCR-based method could be used to screen positive KO
296 colonies, not relying on expensive genotyping methods such as high-throughput next generation
297 sequencing. Finally, we selected three independent mutant lines for each *ptACSL* single mutants for
298 further phenotyping.

299 **Growth and lipid content of *ptACSL* knockout mutants**

300 To evaluate the influence of mutations on cell growth and lipid accumulation, we carried out
301 time-course growth experiments using single mutants for each *ptACSL* gene under both static culture

302 and air-bubbling conditions. During both 12 days of static cultivation and 9 days of growth with air-
303 bubbling, there was no difference in cell density, nitrate concentration in the media, and neutral lipid
304 content revealed by Nile Red fluorescence intensity between WT and either *ptACSL1*, *ptACSL2* or
305 *ptACSL4* mutants (Figs 5a, 5b, S3, S4). Interestingly, in static condition, there was a significant
306 increase in neutral lipid content in the *ptACSL3* mutants since day 7, at which nitrate concentration
307 was reduced to as half as the initial concentration (Figs 5b, 5c). The *ptACSL3* mutants accumulated
308 more TAGs compared with WT, which was revealed by a thin-layer chromatography (TLC) analysis
309 of lipids from these algal samples (Fig.5d). We further evaluated the growth of the mutants under
310 lower or higher temperature, and found there was no difference in growth between WT and the
311 *ptACSL3* mutants at 15°C in static culture (Fig.S5a). By contrast, WT cells grew slowly when the
312 temperature was increased to 28°C while the growth of the *ptACSL3* mutants was almost arrested
313 (Fig.S5b). However, when algal cultures were bubbled with air (low CO₂, ~0.04% vol/vol CO₂),
314 cell densities of both *ptACSL1* and *ptACSL3* mutants were significantly reduced compared with WT
315 (Fig.5e), especially after 6 days of growth. Indeed, while nitrate in the F/2 media in WT was almost
316 exhausted at day 6, nitrate concentration in the *ptACSL3* mutants was reduced to the lowest level at
317 day 8 (Fig.5f). Therefore, neutral lipid content was significantly reduced in the *ptACSL3* mutants
318 after day 7 in comparison with WT, maybe because nitrogen starvation starts a bit later in *ptACSL3*
319 mutants. TLC also showed that the *ptACSL3* mutants accumulated less TAGs, especially at day 7
320 (Figs 5g, 5h). In bubbled air condition, we observed a slower growth of the *ptACSL5* knockdown
321 mutants compared with WT from day 4 and nitrate was almost exhausted at day 5 for both WT and
322 the mutants. However, the impairment of *ptACSL5* did not result in a change of neutral lipid content,
323 which was revealed by Nile red staining (Fig.S6).

324 **ptACSL3 mutation affects TAG synthesis and fatty acid profiles of glycolipids when** 325 **algal culture is bubbled with air**

326 FAs and lipids from WT and the *ptACSL3* mutants were analyzed at the exponential (6 d) and
327 stationary phases (9 d) of growth. When cells were bubbled with filtered air and harvested at dusk,
328 total FA accumulation in the exponential phase was decreased from 738 nmol mg⁻¹ of dry cell
329 weight (dcw) in WT to 592 nmol mg⁻¹ of dcw in the *ptACSL3* mutants. In the stationary phase,
330 total FA content declined from 1137 nmol mg⁻¹ of dcw in WT to 811 nmol mg⁻¹ of dcw in the
331 *ptACSL3* mutants. KO of *ptACSL3* significantly reduced total FA accumulation relative to WT,

332 indicating that *de novo* FA synthesis is inhibited in the *ptACSL3* mutants. As the major FAs
333 synthesized in the chloroplast, palmitic acid (16:0) and palmitoleic acid (16:1) showed
334 significantly reduced accumulation. The level of 16:0 was reduced from 132 nmol mg⁻¹ of dcw in
335 WT to an average of 89 nmol mg⁻¹ of dcw in the exponential phase, and from 331 to 189 nmol mg⁻¹
336 of dcw in the stationary phase. Similarly, the level of 16:1 was also decreased in the mutants at
337 both growth phases (Fig.6a). KO of *ptACSL3* did not change the accumulation of other FA
338 including the abundant EPA. Analysis of FA profiles revealed a significant decrease in the
339 proportions of 16:0, 16:1 and 18:1 balanced by an increase in 16:2, 16:3 and 20:5 in the *ptACSL3*
340 mutants relative to WT at both growth phases (Fig.6b).

341 Glycerolipidomic analysis was performed to determine how the inactivation of *ptACSL3*
342 influenced lipid class composition and the distribution of different molecular species within each
343 lipid class. Compared to WT, disruption of the *ptACSL3* gene led to a significant reduction of TAG
344 (76%, p<0.0001), DAG (50%, p<0.01) and DGTA (46%, p<0.05) and an increase in every
345 phospholipid, i.e. phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine
346 (PE) and phosphatidylcholine (PC) in the exponential phase (Fig.7a). With the depletion of
347 nitrogen, WT accumulated large amounts of TAGs in the stationary phase, accounting for 48% of
348 total glycerolipids. However, the relative content of TAG was reduced to 25% in the *ptACSL3*
349 mutant (48% reduction, p<0.001) (Fig.7a). In addition to TAG, the *ptACSL3* mutants also showed
350 lower DAG content (51% reduction at the exponential phase, p<0.01; 43% reduction at the
351 stationary phase, p<0.01). The decrease in the proportions of DAG and TAG in the *ptACSL3*
352 mutants was compensated by a significant increase in PC (5-fold increase, p<0.0001) and three
353 abundant thylakoid glycolipids (55% increase in SQDG, p<0.05; 47% increase in MGDG,
354 p<0.0001; and 78% increase in DGDG, p<0.001) in the stationary phase (Fig.7a).

355 Further quantification of molecular species showed that at both growth phases, both MGDG
356 and DGDG in the *ptACSL3* mutants had a significant decrease in the proportions of molecular
357 species containing 16:0/16:0 and 16:1/16:0. In MGDG of the *ptACSL3* mutants, 16:0/16:0 and
358 16:1/16:0 represent two species in which 16:0 is mainly enriched; 16:1 is distributed in seven
359 molecular species, among which four species (14:0/16:1, 16:1/16:0, 16:1/16:2, and 16:1/18:1)
360 showed significantly decreased levels while others (16:1/16:1, 16:1/16:3, and 20:5/16:1) remained
361 unchanged at the stationary phase (Fig.7b, Table S1). DGDG contains mainly nine molecular

362 species and 16:0 is esterified in three species. Although the level of 20:5/16:0 was slightly
363 increased in the *ptACSL3* mutants, 16:0/16:0 and 16:1/16:0 that contained the highest proportion
364 of 16:0 showed a pronounced decrease (Fig. 7c, Table S1). These results suggest that the most
365 abundant galactoglycerolipids MGDG and DGDG in the *ptACSL3* mutants had less 16:0 and 16:1,
366 which is also supported by the analysis of FA composition of these two galactoglycerolipids (Figs
367 S7, S8). As molecular species of MGDG and DGDG containing 16:0/16:0 and 16:1/16:0 in their
368 DAG backbone are thought to be derived from the prokaryotic pathway, the impairment of
369 *ptACSL3* led to a pronounced alteration of FA profiles of chloroplast membrane lipids. In the
370 plastid, 16:0 and 16:1 are released as acyl-ACPs from the FA synthase, and then transferred to
371 glycerol backbone of galactoglycerolipids. This process does not require the activated form of
372 acyl-CoA thioesters. Therefore, the reduction of 16:0 and 16:1 in two galactoglycerolipids in the
373 *ptACSL3* mutants reflects an inhibition of plastidic *de novo* FA synthesis. In the *ptACSL3* mutants,
374 MGDG contained more 16:2/16:3, 16:3/16:3, 20:5/16:2, 20:5/16:3, and 20:5/16:4, while DGDG
375 had higher levels of 20:5/16:0, 20:5/16:1, 20:5/16:2, and 20:5/16:3 at the stationary phase (Figs
376 7b, 7c, Table S1). This change suggests that the chloroplast membrane lipids in the mutants
377 contained more polyunsaturated FAs when compared with WT. More than ten FAs were found to
378 be at the *sn*-2 position of PC, where 16:0 was enriched in two molecular species (PC16:1/16:0 and
379 20:5/16:0) and 18:1 was found in the other two species (PC16:1/18:1 and 18:2/18:1). The
380 proportions of all these four molecular PC species and one species having two C18 acyl chains
381 (18:2/18:2) were significantly/slightly reduced in the *ptACSL3* mutants at both growth phases
382 (Fig. 7d, Supporting information Dataset S1), which was compensated by an increase in 16:1/16:1
383 and 20:5-containing molecular species (20:5/16:1, 20:5/18:2, 20:5/18:3 and 20:5/22:6). Taken
384 together, in air bubbled condition, the impairment of *ptACSL3* led to a significant reduction of
385 TAG accumulation, an increase in phospholipid content and a strong alteration in FA profiles of
386 MGDG, DGDG and PC with reduced distribution of 16:0, 16:1 and 18:1 in these lipid classes.

387 To examine whether molecular species of TAG was affected in the *ptACSL3* mutants, we
388 used LC-MS to analyze TAG composition of the WT and mutants grown under static condition
389 and in the bubbling of air. A heat map showing the variation of percentage abundance of 20 major
390 TAG species as the effect of *ptACSL3* disruption under both conditions revealed that the levels of
391 three most abundant TAG species accounting for over 10% of total TAG species (48:1

392 (16:0/16:0/16:1), 48:2 (16:0/16:1/16:1) and 48:3 (16:1/16:1/16:1)) were not changed. When algal
393 cultures were bubbled with filtered air, the levels of two molecular TAG species, including 50:1
394 (16:0/18:1/16:0) and 50:2 (16:1/18:1/16:0), and all 20:5/22:6-containing molecular species were
395 decreased in the *ptACSL3* mutants compared with WT (Fig.8a). The decrease in percentage
396 abundance of 18:1- and 20:5-containing TAG species in the *ptACSL3* mutants was in accordance
397 with the substrate selectivity of *ptACSL3* isozyme towards these two FAs from *in vitro* enzyme
398 assay (Table 1). In the static culture, the *ptACSL3* mutants accumulated more TAGs than WT with
399 no reduced growth rate. However, TAG profiling showed that there was also no difference in
400 percentage abundance in three most abundant TAG species (48:1, 48:2 and 48:3) between the
401 *ptACSL3* mutants and WT, and the levels of only three molecular species accounting for less than
402 3% of total TAG species, TAG 50:3 (16:1/18:1/16:1), 50:4 (16:1/16:1/18:2) and 50:5
403 (16:1/16:1/18:3) were increased in the *ptACSL3* mutants (Fig.8b).

404 **Impacts of the *ptACSL3* KO on transcriptional change upon the transition of growth state**

405 Because the *ptACSL3* mutants showed different change of TAG accumulation under static
406 culture (slow growth state) and in the culture bubbled with filtered air (rapid growth state), we
407 performed mRNA-seq analysis to obtain mechanistic insight into the transcriptome response of
408 *Phaeodactylum* to the shift of growth state. The global gene expression of *Phaeodactylum* as a
409 function of time (i.e., over three time points, 2, 3, and 4 d) under the bubbling of air and static
410 condition (0 d) was measured by mRNA-seq. A total of 5,622 genes, accounting for 45% of all the
411 annotated genes in the *Phaeodactylum* genome
412 (http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index/), were aligned with at least
413 one read (FPKM [Fragments Per Kilobase of transcript per Million mapped reads] value >10) in
414 each biological sample (Supporting information Dataset S2). Based on the definition of differential
415 gene expression, 746 genes were found to be significantly regulated at more than one time point in
416 either WT or the mutant sample (Dataset S2). Growth state transition led to strong transcriptomic
417 responses, which was revealed by the gradually increased numbers of differentially expressed genes
418 (DEGs) during the growth in air-bubbling cultures. Compared with the static culture (0 d), 1937,
419 2543, and 3830 genes were significantly regulated ($P < 0.05$) at 2 d, 3 d, and 4 d respectively in WT;
420 while 2986, 3342, and 3913 genes were detected to be differentially expressed at 2 d, 3 d, and 4 d
421 respectively in the *ptACSL3* mutants. We used these DEG datasets to perform GO analysis with the

422 *Phaeodactylum* genome as the reference, and found that the most obvious difference in the enriched
423 GO terms between WT and the *ptACSL3* mutant was observed at 2 d. In WT, these DEGs were
424 mainly enriched for cell division and structural constituent of ribosome at 2 d; whereas in the
425 *ptACSL3* mutants the most over-represented GO term was “mitochondria” (Figs 9a, 9b).

426 In the mitochondria of most eukaryotes, both tricarboxylic acid cycle and FA β -oxidation occur,
427 which play a central role in the catabolism of organic fuel molecules such as glucose, FAs and amino
428 acids. Mitochondria contain the full enzymatic machinery to oxidize FAs. After the conversion of
429 FAs into CoA esters by a LACS enzyme, the acyl-CoA esters can enter the β -oxidation spiral
430 starting via the well-established cascade of four steps involving dehydrogenation, hydratation,
431 dehydrogenation again and thiolytic cleavage of the acyl-CoA esters. We further determined if the
432 expression patterns of genes associated with mitochondrial FA β -oxidation were affected in the
433 *ptACSL3* mutants. In WT, genes encoding carnitine *O*-acyltransferase (CAT, Phatr3_J859), enoyl-
434 CoA hydratase/3-hydroxyl-CoA dehydrogenase (HADA, Phatr3_J35240) and β -ketoacyl-CoA
435 thiolase (KCT3, Phatr3_J28068) known as the mitochondrial trifunctional protein β -subunit, were
436 induced after growth state transition. However, the expression of these genes was downregulated or
437 remained unchanged (Fig.9c). Moreover, the expression of genes encoding carnitine *O*-
438 acyltransferase (CAT2, Phatr3_J48078), acyl-CoA dehydrogenase (MACAD1, J11014) catalyzing
439 the first step in β -oxidation, and 3-hydroxyacyl-CoA dehydrogenase (HAD2, Phatr3_J39681) was
440 slightly downregulated in WT after transfer to the culture bubbled with air, but the degrees of
441 downregulation were higher in the *ptACSL3* mutants. These results indicate that disruption of
442 *ptACSL3* led to an inhibition of mitochondrial FA β -oxidation upon transition from slow to rapid
443 growth. As mitochondrial β -oxidation generates acetyl-CoAs and reducing power and is essential
444 for FA degradation and maintaining energy homeostasis in the cell, inhibition of mitochondrial β -
445 oxidation might have a negative influence on FA synthesis under air-bubbling condition, which
446 caused reduced FA content and TAG synthesis in the *ptACSL3* mutants (Fig.5h, Fig.6a).

447

448 **Effect of the *ptACSL1/ptACSL5* mutation on lipid synthesis**

449 As the expression of *ptACSL1* was up-regulated during nitrogen deprivation, we analyzed lipid
450 samples prepared from WT and the *ptACSL1* mutants at the exponential phase (0 h) and deprived
451 of nitrogen for 72 h (72 h). There was no difference in FA composition between WT and the *ptACSL1*

452 mutant, except a small decrease in 20:5 at exponential phase in the mutant (Fig.S9). This decrease
453 could be explained by a global decrease in 20:5-containing TAG and polar lipid species (Dataset
454 S1). At 72 h of nitrogen starvation, membrane lipids apart from DAG were more affected in the WT
455 than in the *ptACSL1* mutant, suggesting *ptACSL1* might be involved in membrane lipid remodeling
456 during nitrogen starvation.

457 The FA profiles and content of WT and the *ptACSL5* mutants were analyzed in the exponential
458 and stationary phase of growth (bubbled with air). It revealed a slight increase in total FA content
459 of the *ptACSL5* mutants at the stationary phase (WT, 766±55, and *ptACSL5*, 867±67 nmol mg⁻¹dcw),
460 which was correlated by a slight increase in the levels of 16:1 and proportions of 16:1 in FA profiles
461 at both phases (Fig.S10). Glycerolipidomic analysis showed that 16:1 was highly enriched in the
462 *sn-1* position of PC, and the levels of almost all 16:1-containing molecular species were increased
463 in PC of the *ptACSL5* mutants in the exponential phase at the expense of a reduction of 20:5-
464 containing species (Dataset S1). In the *ptACSL5* mutants, 16:1-containing TAG species derived from
465 prokaryotic pathway were increased, which was balanced by a slight decline of TAG 20:5-
466 containing species and TAG 16:0/16:0 species in both phases (Dataset S1). To further test whether
467 peroxisomal *ptACSL5* is involved in FA β-oxidation, WT and the *ptACSL5* mutants were
468 maintained in N-free medium for 3 d to promote TAG accumulation. After that period, algal cultures
469 were transferred to N-replete medium for additional 4 d to induce the turnover and hydrolysis of
470 storage lipids. The *ptACSL5* mutants still showed reduced growth rate compared with WT during
471 both N starvation and resupply, but these algal strains displayed similar Nile red fluorescence
472 (Fig.S11), indicating that storage lipid catabolism after a shift from N-starved to N-replete medium
473 was not impaired in the mutants. Our results support that peroxisomal FA β-oxidation is not
474 correlated to storage lipid remobilization in *Phaeodactylum* but may be involved in the recycling of
475 long-chain to very-long-chain FAs derived from polar lipids (Jallet et al. 2020). To conclude,
476 *ptACSL5* might be involved in the balance of 16:0 versus 16:1 in acyl-CoA fluxes for membrane
477 lipids (PC) and TAG synthesis, targeting a part of 16:0 into peroxisomal β-oxidation to prevent it
478 from further elongation to enter the 16:0-to-20:5 synthetic route.

479

480 Discussion

481 *Phaeodactylum* accumulates large amounts of TAGs and produces EPA up to 30% of total

482 FAs with trace amounts of C18 FA intermediates, representing a particularly interesting model for
483 studies on VLC-PUFA and lipid biosynthesis. Despite recent advances in understanding the main
484 routes of lipid metabolism in *Phaeodactylum* (Smith et al. 2021), little is known about FA
485 activation, transport between different subcellular compartments, and flux governing the ultimate
486 deposition of VLC-PUFA into galactoglycerolipids. Consistent with high proportions of palmitic
487 acid (16:0), palmitoleic acid (16:1n-7) and EPA (20:5) in total FAs, palmitic, palmitoleic and
488 EPA-CoA were detected as the most abundant species in acyl-CoA profiles of *P. tricornutum* cells
489 (Hamilton et al. 2014). These high levels of acyl-CoAs may potentially act as acyl donors or
490 intermediates for VLC-PUFA synthesis and TAG assembly. *P. tricornutum* has at least five LACS
491 isozymes catalyzing the formation of acyl-CoAs from free FAs and CoA. Recently, ptACSL4 was
492 identified as one of lipid droplet-associated proteins in the proteome analysis (Lupette et al. 2019).
493 However, in our study the *ptACSL4* mutants did not show any lipid-associated phenotype under
494 the growth conditions. This finding is in contrast to its ortholog of the heterokont *Microchloropsis*
495 *gaditana*. ptACSL4, also called PtACSBG (Phatr3_J45510), was recently identified as the LACS
496 isoform of the bubblegum subfamily and shares five conserved ACSBG motifs with MgACSBG,
497 the phylogenetically closely related LACS isoform of the bubblegum type in the eustigmatophytes
498 *M. gaditana* (Billey et al. 2021). In this oleaginous microalga, the *MgACSBG* mutant of point
499 mutations led to an overall reduction of 16:1 in lipids and C20 acyls in the betaine lipid,
500 supporting a critical role in the generation of 16:1-CoA and 18:3-CoA within the cell. We did not
501 evaluate these two molecular species, but the data we obtained with tested acyl-CoA substrates,
502 showing that ptACSL4 was active exclusively with 18:1-CoA and 20:5-CoA, and inactive when
503 provided with saturated acyl-CoAs (Table 1), are consistent with a specific function in the
504 activation of unsaturated molecular species, similar to that reported in *M. gaditana*.

505 ptACSL1 is the only LACS isozyme located in the outermost of cER membrane with the C-
506 terminus facing the cytoplasm (Fig.1), indicating that it may be the most possible isozyme
507 responsible for activating *de novo*-synthesized FAs exported from the plastid. During nitrogen
508 starvation, membrane lipids seem to be protected from degradation or recycling in the *ptACSL1*
509 mutants compared to WT. In *Arabidopsis*, of the nine LACS isoforms only LACS9 was showed to
510 reside in the plastid envelop and it might be the major LACS isoform involved in the export of
511 plastidial FAs for TAG formation. Although the *lacs9* null mutant had a 90% reduction of

512 chloroplast LACS activity, the mutant did not exhibit any detectable phenotype. Further studies
513 revealed that ER-localized LACS1 showed overlapping functions with LACS9 in TAG biosynthesis
514 (Zhao et al. 2010). In our study, ptACSL1 displayed LACS activity towards all tested FAs and its
515 disruption had no impact on the amounts of TAG (Fig.5), which is mainly constituted of 16:0 and
516 16:1, suggesting one or more additional LACS isozymes may be involved in the activation of
517 plastid-exported FAs and contribute to acyl-CoAs for storage TAG synthesis.

518 We identified the mitochondrial ptACSL3 as the LACS isozyme contributing to TAG synthesis.
519 The *ptACSL3* mutants showed distinct TAG-associated phenotypes when cells were bubbled with
520 air and in static culture (Figs 5d, 5h). When algal cells are exposed to rapid changes in nutrient
521 availability, temperature and light conditions, they must rapidly degrade or remodel storage and
522 membrane lipids to ensure growth and survival. FA degradation thus plays an important role in the
523 response to these changing conditions. Recently, Jallet et al. (2020) confirmed the existence of a
524 functional mitochondrial β -oxidation pathway that plays a principle role in the liberation of energy
525 from TAG-derived FAs in *P. tricornutum*. This pattern differs in the green alga *Chlamydomonas* that
526 possesses the major reactions of FA β -oxidation in peroxisomes (Kong et al. 2017). In our study,
527 when algal cultures were bubbled with filtered air, in rapidly growing cells mitochondrial FA β -
528 oxidation plays a 'housekeeping' role in removing harmful free FAs which may be derived from the
529 degradation of acyl-lipids. Disruption of *ptACSL3* can result in the impairment of FA β -oxidation
530 that breaks the balance of keeping the amounts of cytosolic free FAs coming from phospholipids
531 below the threshold and also led to a pronounced inhibition of plastidial *de novo* FA synthesis, which
532 produces less FA acyls for TAG and chloroplast lipid synthesis and then reduced TAG content in the
533 *ptACSL3* mutants. By contrast, algal cells grown in static culture may not need a highly dynamic
534 and active mitochondrial FA β -oxidation to remove free FAs. Thus, we observed that a similar
535 growth of the *ptACSL3* mutants to WT under the static condition (Fig.5a), which may ensure that
536 the mutant could accumulate more TAGs through diverting a part of phospholipid derived FAs from
537 mitochondrial β -oxidation to the TAG synthesis pathway in the absence of ptACSL3.

538 In *Phaeodactylum*, free FAs may be derived from multiple routes: (1) *de novo* synthesis in the
539 plastid, then hydrolysis by acyl-ACP thioesterase from ACP and finally export outside the plastid;
540 (2) turnover or recycling of membrane lipids (e.g. DGTA and PC) under some conditions such as
541 nitrogen or carbon starvation; (3) TAG remobilization, possibly involving TAG lipases that remove

542 acyl chains from glycerol backbone of TAG. Of the five LACSs, ptACSL1 is localized in the
543 outermost of cER membrane and displayed the highest catalytic efficiency (k_{cat}/K_m) for 20:5 in our
544 *in vitro* enzyme assay (Table 1). In addition, the *ptACSL1* mutants grown in both the exponential
545 phase (0 h) and N-deprived condition (72 h) showed slight decrease in the levels of 20:5-containing
546 species in the plastid glycolipid of MGDG (Dataset S1), suggesting a possible role in transfer of
547 ER-derived 20:5 to plastid membranes. ptACSL3 and ptACSL5 may play a role in generating acyl-
548 CoAs that are channeled into mitochondria or peroxisome for β -oxidation (Fig.10). How ptACSL2
549 and ptACSL4 are involved in lipid metabolism in *Phaeodactylum* remains to be further elucidated.
550 Since the *Phaeodactylum* genome encodes multiple LACS isoforms, functional redundancy
551 possibly exists within the *lacs* gene family. To distinguish the contributions of each ptACSL to lipid
552 metabolism, generation of higher-order *lacs* KO mutants using multiplexed CRISPR/Cas9 method
553 will provide more insights into the precise roles of each LACS and uncover the complex network
554 of functions of all LACS isozymes in *Phaeodactylum*.

555

556

557 **Figure legends**

558 **Figure 1.** In vivo localization of *Phaeodactylum* LACS isoforms. Self-assembly GFP system was
559 used for analysis of ptACSL1 plastid localization. The (c)ER-characteristic fluorescence pattern
560 could be observed when ptACSL1 fused to GFP_S11 was simultaneously expressed with a cytosolic
561 GFP_S1-10. Expression of ptACSL1_S11 with the ER marker (PDI) fused to GFP_S1-10 led to no
562 fluorescence. Expression of full-length ptACSL2 and ptACSL3 with C-terminally fused eGFP
563 indicated the localization in plastid. Localization of ptACSL4:eGFP fusion led to indistinct results
564 (plastid, plasma membrane and cytosol). eGFP with N-terminal fusion with ptACSL5 showed the
565 fluorescence signal in the peroxisome. TL, transmitted light; PAF, plastid autofluorescence; eGFP,
566 enhanced green fluorescence protein; PAF/eGFP, overlap of plastid and GFP fluorescence. Scale bar
567 represents 10 μ m.

568 **Figure 2.** Expression patterns of *Phaeodactylum* LACS family proteins. Lysates of *Phaeodactylum*
569 cultures under nitrogen deprivation condition (A) and supplied with filtered air (low CO₂, ~0.04%
570 CO₂) for 2 h (a2h) and 6 h (a6h) or high concentrations of CO₂ (1%, 4% and 10%) for 2 h and 6 h
571 (B) were analyzed by Western blotting using polyclonal antibodies against ptACSL1-5. Loading of

572 protein samples was equalized using total protein extracts of each sample visualized with coomassie
573 brilliant blue (CBB) staining as loading control.

574 **Figure 3.** Multiple targeted mutagenesis of *ptACSL1-5* genes by CRISPR/Cas9 in *Phaeodactylum*.
575 (A) Schematic depiction of the multiple CRISPR/Cas9 method for simultaneously targeting multiple
576 sites in the *Phaeodactylum* genome. Each of the CRISPR-episome plasmids consists of three
577 different gRNA expression cassettes driven by the U6 promoter, Cas9 protein, the *ShBle* resistance
578 gene cassette, and the yeast-derived *CEN6-ARSH4-HIS3* element conferring episomal replication in
579 the diatom *Phaeodactylum*. The gRNAs are transcribed independently and direct Cas9 to multiple
580 targets. (B) Frequencies of different editing events observed in *ptACSL1-5* target regions induced
581 by a single gRNA or double gRNAs using the multiple CRISPR/Cas9 system, which were calculated
582 from 30 mutated sites (not including those of homozygous fragmental deletions between two
583 targeted sites). (C) Comparison of mutation frequencies of large fragment deletions between two
584 targeted sites occurred among five *ptACSL* genes.

585 **Figure 4.** Genome editing of *ptACSL1* and *ptACSL3* using multiple CRISPR/Cas9 system led to the
586 generation of fragmental deletions in *Phaeodactylum*. Schematic representations show the
587 fragmental deletions between two targeted sites of the *ptACSL1* (a) and *ptACSL3* (b) mutants. A
588 pair of primers was designed to detect the large fragment deletions induced by multiple
589 CRISPR/Cas9 in the *ptACSL1* (c) and *ptACSL3* (d) mutants with PCR using genomic DNAs and
590 total complementary DNAs (cDNAs) as templates. For DNA sequencing, PCR amplifications were
591 carried out using primer pairs flanking the designed target sites, and the PCR products were
592 sequenced directly using internal specific primers. Representative sequences of fragment deletions
593 in the *ptACSL1* (e) and *ptACSL3* (f) mutants were aligned with that of wild type. The PAM region
594 is shown in blue color letters, and the number at the end shown in red color letters indicates deleted
595 (-) bases between two Cas9 cuts.

596 **Figure 5.** Phenotype analyses of the *ptACSL1* and *ptACSL3* mutants. (a) Growth curves of the
597 *ptACSL1* and *ptACSL3* mutants incubated in static cultures for 12 days. Cell densities were
598 determined with a Z2 Coulter Counter. Results are the average of three biological replicates
599 (independent lines) with error bars indicating standard deviations ($n=3$). (b) Nitrate concentrations
600 in culture media for the *ptACSL1* and *ptACSL3* mutants incubated in static cultures. (c) Nile red
601 fluorescence intensities of the *ptACSL1* and *ptACSL3* mutant cells incubated in static cultures for

602 12 days. Statistical analysis using paired-sample Student's *t* test was performed by GraphPad Prism
603 (v9.0): *, $P < 0.05$; **, $P < 0.01$. (d) A thin-layer chromatogram of total lipids from wild type and the
604 *ptACSL3* mutants incubated in static cultures for 9 d, 10 d, 11 d, and 12 d. Each lipid sample was
605 extracted from $\sim 5 \times 10^7$ cells. TAGs were visualized with 0.01% (w/v) primuline reagent. (e) Growth
606 curves of the *ptACSL1* and *ptACSL3* mutants under air bubbling condition for 9 days. Data are the
607 average of three biological replicates (independent lines) with error bars indicating standard
608 deviations ($n=3$). (f) Nitrate concentrations in culture media for the *ptACSL1* and *ptACSL3* mutant
609 cultures bubbled with filtered air. (g) Nile red fluorescence intensities of the *ptACSL1* and *ptACSL3*
610 mutant cultures bubbled with filtered air for 4-9 days. Statistical analysis using paired-sample
611 Student's *t* test was performed by GraphPad Prism (v9.0): *, $P < 0.05$; **, $P < 0.01$. (h) A thin-layer
612 chromatogram of total lipids from wild type and the *ptACSL3* mutants under air-bubbling condition.
613 1, 3, 5, 7, lipid samples from wild type grown for 7d, 8 d, 9 d, and 10 d, respectively; 2, 4, 6, 8, lipid
614 samples from the *ptACSL3* mutant grown for 7d, 8 d, 9 d, and 10 d, respectively. Each lipid sample
615 was extracted from $\sim 5 \times 10^7$ cells, and TAGs were visualized with 0.01% (w/v) primuline reagent.

616 **Figure 6.** Comparative analysis of total fatty acid content (a) and fatty acid composition (b) (Mol
617 of total fatty acids) of wild type and the *ptACSL3* mutants grown at exponential and stationary
618 phases.

619 **Figure 7.** Compared analysis of the various glycerolipids (a) and lipid molecular species (b, MGDG;
620 c, DGDG; d, PC) between wild type and the *ptACSL3* mutants grown at the exponential and
621 stationary phases. Values are means of three biological replicates. *Asterisk indicates significant
622 difference between wild type and the mutant as determined by the Student's *t*-test. *, $P < 0.05$,
623 ** $P < 0.01$.

624

625 **Figure 8.** The disruption of *ptACSL3* affects molecular composition of TAG. LC-MS was used to
626 quantify different molecular species of TAG in wild type and the *ptACSL3* mutants in three
627 biological replicates. A heat map shows the percentage abundance of 20 major TAG species in each
628 biological replicate relative to the average percentage abundance of that species in wild type. Algal
629 cultures were bubbled with filtered air and samples were harvested at day 7 (a), or cells were grown
630 under static culture condition and harvested at day 9 (b) for lipid extraction and TAG profiling.

631 **Figure 9.** Transcriptional change of the *ptACSL3* mutants upon the shift from static culture to

632 cultures bubbled with filtered air. GO functional enrichment analysis of differentially expressed
 633 genes in wild type (a) and the *ptACSL3* mutants (b) upon the shift from static culture (0d) to culture
 634 bubbled with air for 2 d. Biological processes are listed on the Y-axis with their enrichment folds
 635 against all *Phaeodactylum* genes (reference) presented on the X-axis. c, for each differentially
 636 expressed gene, fold changes (FC) are presented as heat maps with shades of red or green colours
 637 according to the scale bar. Fold change was calculated as $\log_2(\text{FPKM (Dx, BA)}/\text{FPKM (0d, SC)})$
 638 (FPKM=absolute abundance of transcripts, Dx=a time point: 2d/3d/4d; BA=in culture bubbled with
 639 air; SC=in static culture). Changes of genes with FDR-corrected P value <0.05 were considered as
 640 statistically significant. Time refers to the duration (in days) since the transfer of algal cells from
 641 static culture to the cultures bubbled with filtered air. Data are the mean of the FC of 2d vs 0d, 3d
 642 vs 0d and 4d vs 0d of wild type and the *ptACSL3* mutants. Data are presented in [Table S2](#).

643 **Figure 10.** Predicted LACS-mediated lipid metabolic pathways in *Phaeodactylum*. *De novo* fatty
 644 acid (FA) synthesis takes place in the plastid. 16:0-ACP is released from the fatty acid synthase,
 645 and then enters the prokaryotic pathway to be desaturated by soluble acyl-ACP desaturases to
 646 produce C16 FAs with one or more double bonds which are used for synthesis of MGDG, DGDG,
 647 SQDG and PG as chloroplast membrane lipids. The majority of 16:0 and 16:1 in the plastid is
 648 exported to the cytosol, converted to 16:0-CoA and 16:1-CoA and enters the Kennedy pathway for
 649 TAG biosynthesis. In the cytosol, 16:0-CoA can be elongated to 18:0-CoA and further desaturated
 650 to produce 18:1 Δ 9-CoA. This FA enters long-chain polyunsaturated FA synthesis pathway to
 651 produce 20:5 (EPA) and 22:6 via the combined n3 and n6 pathways. EPA can be imported back
 652 into the plastid via the unknown omega-3 pathway for the synthesis of chloroplast membrane
 653 lipids. Some free FAs, mainly 16:0 and 16:1 species, can be released from TAG hydrolysis, then
 654 activated by LACS enzymes and transported into mitochondria or peroxisomes for β -oxidation.
 655 Enzymes are shown in red: PAD, palmitoyl-ACP Δ 9-desaturase; FAD, fatty acid desaturases;
 656 Des6, plastidic soluble Δ 6-desaturase; MGD, MGDG synthase; DGD, DGDG synthase; SQD2,
 657 SQDG synthase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid
 658 acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase. G3P,
 659 glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerols;
 660 TAG, triacylglycerols; MGDG, monogalactosyldiacylglycerol; SQDG,
 661 sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; DGTA, diacylglycerol-hydroxymethyl-

662 *N,N,N*-trimethyl- β -alanine; FFA, free fatty acid; OAA, oxaloacetate; TCA, tricarboxylic acid; ER,
663 endoplasmic reticulum; FASII, fatty acid synthase II.

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665

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674

675 **Authors' contributions**

676 XH, WC, A. Amato, DM, HJ and LY performed experiments. JJ, EM, HH and FH analyzed data.
677 MM and A. Allen contributed to the generation of *ptACSL* mutant lines by multiplexed
678 CRISPR/Cas9 method. YG planned and designed the research and wrote the manuscript. XH and
679 WC contributed equally.

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