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1 **Two plastidial lysophosphatidic acid acyltransferases differentially**
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43

44 **Summary**

- 45 • Lysophosphatidic acid acyltransferases (LPAATs) catalyze the formation of phosphatidic
46 acid, a central metabolite in both prokaryotic and eukaryotic organisms for glycerolipid
47 biosynthesis. *Phaeodactylum tricornutum* contains at least two plastid-localized LPAATs
48 (ptATS2a and ptATS2b), but their roles in lipid synthesis remain unknown.
- 49 • Both ptATS2a and ptATS2b could complement the high temperature sensitivity of the
50 bacterial *plsC* mutant deficient in LPAAT. *In vitro* enzyme assays showed that they prefer
51 lysophosphatidic acid over other lysophospholipids.
- 52 • ptATS2a is localized in the plastid inner envelope membrane and
53 CRISPR/Cas9-generated *ptATS2a* mutants showed compromised cell growth,
54 significantly changed plastid and extra-plastidial membrane lipids at nitrogen-replete
55 condition and reduced triacylglycerols (TAGs) under nitrogen-depleted condition.
56 ptATS2b is localized in thylakoid membranes and its knockout led to reduced growth rate
57 and TAG content but slightly altered molecular composition of membrane lipids.
- 58 • The changes of glycerolipid profiles are consistent with the role of both LPAATs in the
59 *sn*-2 acylation of *sn*-1-acyl-glycerol-3-phosphate substrates harboring 20:5 at the *sn*-1
60 position. Our findings suggest that both LPAATs are important for membrane lipids and
61 TAG biosynthesis in *P. tricornutum*, and further highlight that 20:5-Lyso-PA is likely
62 involved in the massive import of 20:5 back to the plastid to feed plastid glycerolipid
63 syntheses.

64 **Key words:** Chloroplast membrane lipids, Lysophosphatidic acid acyltransferase, Multiplexed
65 CRISPR/Cas9, *Phaeodactylum tricornutum*, Triacylglycerol.

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69 Introduction

70 Phosphatidic acid (PA) and its dephosphorylated form, diacylglycerol (DAG) are key precursors for
 71 the biosynthesis of membrane glycerolipids and the storage lipid triacylglycerol (TAG) (Miège and
 72 Maréchal, 1999; Okazaki et al. 2006). PA is generated predominantly through two steps of acylation
 73 reactions at the *sn*-1 and *sn*-2 positions of glycerol-3-phosphate (G3P), which are catalyzed by G3P
 74 acyltransferase (GPAT; EC 2.3.1.15) and lysophosphatidic acid acyltransferase (LPAAT; EC 2.3.1.51).
 75 In the second reaction, LPAAT transfers an acyl group to the *sn*-2 position of lysophosphatidic acid
 76 (lysoPA) to produce PA and is essential to PA synthesis in almost all cellular organisms.

77 In *Escherichia coli*, *plsC* encodes the sole LPAAT catalyzing the conversion of lysoPA to form PA,
 78 which is then utilized as substrate for synthesis of membrane phospholipids consisting mainly of
 79 phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL). The *plsC*-deficient
 80 mutant is non-permissive for cell growth when temperature was elevated (Cullinane et al. 2005). In the
 81 yeast *Saccharomyces cerevisiae*, *SLC1*, together with *SLC4*, encodes partially redundant LPAAT
 82 activity. The *slc1Δslc4Δ* double knockout mutant is lethal, suggesting an essential role of LPAAT
 83 activity in yeast.

84 In higher plants, FAs serving as building blocks for all membrane lipids and TAG are exclusively
 85 synthesized in the stroma of chloroplasts. Then, two parallel pathways operate for the biosynthesis of
 86 chloroplast thylakoid lipids, one localized within the plastid, and the other in the endoplasmic
 87 reticulum (ER) (Roughan and Slack, 1982). A portion of the *de novo*-synthesized FAs can be directly
 88 used in the chloroplast for successive acylation of G3P to produce lysoPA and PA, catalyzed by a
 89 plastid acyl-acyl carrier protein (ACP):G3P acyltransferase encoded by *ATS1* (Kunst et al. 1988; Xu et
 90 al. 2006) and a plastid LPAAT encoded by *ATS2* (Kim and Huang 2004), respectively. PA is
 91 subsequently dephosphorylated in the chloroplast to generate DAG, both serving as precursors for
 92 synthesis of the predominant thylakoid membrane lipids, mainly PG from PA via a CDP-DAG
 93 intermediate, and monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and
 94 sufoquinovosyldiacylglycerol (SQDG), from DAG. This chloroplast pathway is referred to as the
 95 “prokaryotic pathway”. In parallel, FAs synthesized in the chloroplast can also be exported to the
 96 cytosol and assembled into extra-plastidial membrane glycerolipids and TAG at the ER. G3P in the ER
 97 is sequentially esterified to generate lysoPA and PA by membrane-bound acyl-CoA:G3P
 98 acyltransferase and LPAAT using acyl-CoA as FA donor, respectively. In this so-called “eukaryotic

99 pathway”, PA generated in the ER is dephosphorylated into DAG, which serves as substrate for the
100 formation of extra-plastidial membrane lipids and TAGs (Benning 2008). In many plants, there is a
101 large flux of glycerolipids between the ER and chloroplast, as lipid precursors assembled at the ER can
102 find their way back to the chloroplast feeding the biosynthesis of thylakoid membrane lipids (Xu et al.
103 2008). It was found that the prokaryotic pathway operating in the chloroplast produces glycerolipids
104 containing mainly 16-carbon (C16) FAs at the *sn*-2 position of DAG backbone, while the same position
105 of glycerolipids synthesized by the ER-located eukaryotic pathway is exclusively occupied by C18 FAs
106 (Heinz and Roughan 1983). This difference in acyl composition of lipids has been observed in many
107 plants and is substantially determined by the distinct substrate specificities and functions of LPAATs or
108 acyl exchange enzymes in the plastid and the ER (Awai et al. 2006).

109 The diatom *Phaeodactylum tricornutum*, a unicellular photoautotrophic microalga with a sequenced
110 genome, is known to produce the ω -3 long-chain polyunsaturated FA, eicosapentaenoic acid (EPA,
111 $20:5\Delta^{5,8,11,14,17}$). Under nutrient stress, *P. tricornutum* accumulates large amounts of TAGs stored in
112 lipid droplet. Diatoms usually have a plastid surrounded by a four-layered membrane system. The
113 outermost two membranes are known as the ‘epiplastidial membrane’ or ‘chloroplast ER’ (cER),
114 possibly deriving from a phagocytic membrane, and the ‘periplastidial membrane’ (PPM) and two
115 innermost membranes corresponding to the ‘classical’ chloroplast envelope (Grosche et al., 2014;
116 Petroustos et al., 2014).

117 The thylakoid membrane lipids from *P. tricornutum* differ strongly from plant lipids. The
118 extremely high proportion of 20:5 at position *sn*-1 in MGDG, DGDG or SQDG (Abida et al, 2015), a
119 FA initially generated in the cytosol (Dolch and Marechal, 2015; Dolch et al, 2017) indicates its
120 massive import back to the plastid, *via* an elusive route tentatively named the ‘omega pathway’
121 (Petroustos et al, 2014). At the *sn*-2 position, MGDG, DGDG and SQDG contain mainly C16 FAs,
122 suggesting that plastid-targeted LPAATs (ATS2s) have very strong preference for a C16-ACP as
123 reported in higher plants (Frentzen et al., 1983). For example, under the nutrient-replete condition the
124 *sn*-2 position of MGDG is exclusively esterified to 16:3 whereas the same position of DGDG is mainly
125 occupied by 16:1 and 16:2. By contrast, storage TAGs that are thought to be assembled at the ER
126 mostly have 16:0 and 16:1 esterified at the *sn*-2 position of their glycerol backbone, indicating that
127 ER-located LPAAT from diatoms may have a preference for C16 similar to that of plastid ATS2(s) as
128 it was also shown in *Chlamydomonas reinhardtii* (Kim et al., 2018). The substrate specificity of the ER

129 LPAAT would thus differ from that of LPAATs characterized in most land plants. Eventually, *P.*
 130 *tricornutum* may lack specific signature that could support the existence of a potential eukaryotic
 131 pathway providing diacyl precursors for the synthesis of chloroplast membrane lipids. Indeed, in plants,
 132 PA and DAG have been suggested as the species transported from the ER to the chloroplast, *via* this
 133 eukaryotic pathway, for MGDG synthesis (Slack et al., 1977; Xu et al., 2005; Lu and Benning, 2009;
 134 Karki et al., 2019). In *P. tricornutum*, whether there is a transfer of a diacyl-moiety such as PA/DAG
 135 from extra-plastidic phospholipids to feed galactoglycerolipid synthesis is still unknown.

136 The understanding of lipid biosynthesis in diatoms and our ability to genetically manipulate some
 137 oleaginous species to produce oils with tailored FA composition and increased TAG content, await the
 138 identification of key enzymes. In the diatom *P. tricornutum*, several acyltransferases involved in TAG
 139 synthesis have been reported and intracellular TAG content could be enhanced by overexpression of a
 140 glycerol-3-phosphate acyltransferase, or a type 2 diacylglycerol acyltransferase (Niu et al. 2016;
 141 Haslam et al. 2020; Wang et al. 2020; You et al. 2023). In this study, we identified Phatr3_J11916 and
 142 Phatr3_J43099 as plastid-localized LPAATs in *P. tricornutum*. We characterized their LPAAT
 143 activities by heterologous expression in bacterial and yeast mutants and determined their subcellular
 144 localization. We further generated single mutants using multiplexed CRISPR/Cas9 method and
 145 characterized their lipid-associated phenotypes under different conditions with high-resolution
 146 lipidomics approaches. We then characterized their roles in membrane and storage glycerolipid
 147 homeostasis in *P. tricornutum*.

148 **Materials and methods**

149 A detailed method section, including plasmid construction, *in vitro* enzyme assay, generation of
 150 *ptATS2* mutants, photosynthetic measurement and glycerolipid extraction and analysis, is
 151 presented in Supporting Information Methods S1.

152 **Microbial strains and growth conditions**

153 *Escherichia coli* DH5 α or EPI300 strain was grown at 37°C in Luria Broth (LB) supplemented
 154 with appropriate antibiotics (ampicillin 50 mg L⁻¹ or gentamycin 10 mg L⁻¹ or tetracyclin 20 mg
 155 L⁻¹). *E. coli* *plsCA* mutant SM2-1 was grown in LB without antibiotic and the complementation strains
 156 harboring the empty vector pQE60 or expressing *ptATS2a/2b* were grown in LB supplemented with 50
 157 mg L⁻¹ ampicillin at 37°C or 42°C. *Phaeodactylum tricornutum* Bohlin (CCMP 2561) from the culture
 158 collection of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow

159 Laboratory for Ocean Sciences, East Boothbay, ME) was grown in artificial sea water enriched with f/2
160 medium (Guillard 1975), at 22°C under cool white fluorescence lights ($75 \mu\text{E m}^{-2} \text{s}^{-1}$) and a
161 photoperiod of 12 h light: 12 h dark. The *P. tricornutum* *ptATS2a* and *ptATS2b* mutants were grown in
162 f/2 medium supplemented with appropriate antibiotics (zeocin 50 mg L^{-1} or nourseothricin 100 mg L^{-1}).
163 For the cell growth experiments reported in Fig.5a-h, 600 mL of algal cultures of wild type and mutants
164 were grown in 1 L flasks with bubbling of filtered air for 12 days at 22°C in a temperature-controlled
165 incubator. In addition, 300 mL of algal cells were grown in 500 mL flasks for 18 days in static culture.
166 In both conditions, algal cultures were inoculated using cultures in exponential phase ($1\text{-}2 \times 10^6$
167 cells/mL) to the same initial cell concentration of approximately 5×10^4 cells/mL and illuminated
168 continuously from $\sim 50 \mu\text{E m}^{-2} \text{s}^{-1}$ cool white fluorescence lights. For the cell growth experiments
169 reported in Fig.5i-l, N-starved cultures were prepared by centrifuging from cultures in exponential
170 phase ($1\text{-}2 \times 10^6$ cells/mL) and washing cells twice in the N-free f/2 media and then resuspending
171 them in N-free media for 4 days. Approximately 5 mL of algal cultures were sampled daily and
172 monitored for cell concentration and nitrate concentration in the media which was measured
173 spectrophotometrically at 220 nm (Collos et al. 1999). The nonpolar lipid content was evaluated by
174 fluorometric assay using the dye Nile Red (Sigma-Aldrich). A total of 6×10^6 cells for each sample
175 were stained with Nile Red and the fluorescence was measured at 572 nm using an F-7000
176 Fluorescence Spectrophotometer (HITACHI, Japan).

177 Generation of *ptATS2a/2b*-GFP fusion transgenic strains and fluorescence microscopy

178 The plasmids carrying *ptATS2a*-GFP or *ptATS2b*-GFP fusion were introduced into *P. tricornutum*
179 cells by microparticle bombardment using the Bio-Rad Biolistic PDS-1000/He Particle Delivery
180 System (Bio-Rad) as described by Zaslavskaja et al. (2000). The zeocin-resistant clones were subjected
181 to a screening based on the observation of detectable GFP fluorescence signals. Subcellular localization
182 of GFP fusion proteins were examined by laser scanning confocal microscopy (Leica SP8).

183 Generation of *P. tricornutum ptATS2a/ptATS2b* mutants

184 The CRISPR/Cas9-episome plasmids harboring multiple sgRNA expression cassettes were transformed
185 into *P. tricornutum* by bacterial conjugation as described by Karas et al. (2015). After 10-14 days,
186 colonies were directly transferred into liquid f/2 media containing $100 \mu\text{g mL}^{-1}$ zeocin in a 48-well
187 plate. The genomic DNA was isolated from each clone and the genomic regions spanning the predicted
188 Cas9 targeting sites were amplified and subjected for DNA sequencing. The Inference of CRISPR

189 Edits tool (ICE, Synthego, <https://ice.synthego.com>) was used to analyze indels resulting from
190 Cas9 targeting of *ptATS2a* or *ptATS2b* by multiple sgRNAs. *P. tricornutum* transformed with the
191 Cas9-2A-sh ble empty vector was used as control strains. Colonies carrying mutations with indel
192 percentage >90% and Knockout Score >90 were selected and grown in f/2 liquid media for cell
193 propagation, and then plated dilutions onto agar plates containing 100 $\mu\text{g mL}^{-1}$ zeocin to obtain single
194 colonies. The genomic DNA from these single colonies was isolated and used as a template for PCR to
195 screen *P. tricornutum ptATS2a* knockdown and *ptATS2b* knockout cell lines. RT-PCR was also
196 conducted for validation of biallelic mutations with large fragment deletion in the obtained mutants.

197 Complementation of *E. coli plsCΔ mutant SM2-1*

198 To test whether the *E. coli plsCΔ mutant (SM2-1) could be complemented by heterologous expression
199 of *ptATS2*, pQE60 (empty vector) or pQE60 harboring *ptATS2a* or *ptATS2b* was transformed into
200 SM2-1 cells. Positive colonies were grown on LB agar plates at 30°C or 42°C to evaluate whether the
201 sensitivity to high temperature of the *E. coli plsCΔ mutant could be rescued.**

202 Heterologous expression of *ptATS2* in the *S. cerevisiae* mutant Y02431

203 The yeast expression plasmids harboring *ptATS2a* or *ptATS2b* or the empty vector (pYES2/CT)
204 were transformed into the yeast mutant Y02431 by the standard lithium acetate procedure (Gietz
205 and Woods, 2002). Transformants were selected by growth on synthetic complete medium (2% glucose
206 [w/v], 0.67% yeast nitrogen base without amino acids) containing appropriate auxotrophic supplements
207 but lacking uracil (SC-Ura). Single colonies were transferred into liquid SC-Ura with 2% glucose and
208 grown at 28°C overnight. To test Lyso-PC sensitivity, each transformant was suspended in sterile
209 distilled water and adjusted to an OD_{600} of 2, 1, 0.5, and 0.1. The resulting 2 μL yeast solution was
210 spotted on an SC-Ura agar plate lacking uracil but supplementing 2% galactose, 1% raffinose and 10 or
211 30 $\mu\text{g/mL}$ lysoPAF. Cell growth was evaluated after 72 h at 28°C to examine the lyso-PC sensitivity
212 for yeast cell growth.

213 In vitro acyltransferase activity assays

214 Lysophospholipid acyltransferase activities were determined by measuring the incorporation of
215 [^{14}C]palmitoyl-CoA or [^{14}C]oleoyl-CoA (acyl donors) into phospholipids in the presence of relevant
216 lysophospholipids (acyl acceptors). Substrate specificity of *ptATS2a* and *ptATS2b* towards the other
217 lysophospholipids were performed with *sn-1-C18:1-lysoPE*, *sn-1-C18:1-lysoPC*, *sn-1-C18:1-lysoPG*,
218 or *sn-1-C18:1-lysoPS*, added to the reaction in equimolar concentration, together with two acyl donors:

219 [¹⁴C]16:0-CoA or [¹⁴C]18:1-CoA.

220 Complementation of *P. tricornutum* ATS2 mutants

221 The coding regions of ptATS2a and ptATS2b were amplified by PCR using primer pairs of
 222 ptATS2aKO_com-F/R and ptATS2bKO_com-F/R, respectively. Oligonucleotides introduced a 5'
 223 *EcoRI* and 3' *SalI* restriction site for cloning the fragment into pPha-Cp1, a modified pPha-T1 plasmid.
 224 In this plasmid, a nourseothricin (NTC) resistance cassette controlled by the FcpD promoter and FcpA
 225 terminator (Slattery et al. 2018) is used to replace the zeocin resistance cassette. The *P. tricornutum*
 226 *ptATS2a* or *ptATS2b* mutated lines were complemented by microparticle bombardment from
 227 logarithmic-phase grown cells with the NTC-resistance plasmid pPha-Cp1 containing the wild-type
 228 ptATS2 sequence. Algal transformants were selected on F/2 agar plates supplemented with 50 µg mL⁻¹
 229 Zeocin and 200 µg mL⁻¹ NTC. Colonies appeared after 3-4 weeks incubation at 22°C with light.

230

231 Results

232 Identification of putative LPAATs in *P. tricornutum*

233 To identify LPAAT enzymes in *P. tricornutum*, the genome of this model diatom
 234 (http://protists.ensembl.org/Phaeodactylum_tricornutum/Info/Index/) was searched with Basic Local
 235 Alignment Search Tool (BLAST), using a query from a partial sequence containing a region conserved
 236 between bacterial, yeast and plant LPAATs. From the results, we examined genes that encoded putative
 237 LPAAT containing a phosphate acyltransferase (PlsC) domain, known to catalyze the transfer of a FA
 238 to the *sn*-2 position of lysoPA to generate PA in bacteria (Schujman and de Mendoza 2008). We also
 239 examined proteins possessing the two conserved motifs (NHX₄D and EGTR), which are highly
 240 conserved in bacteria, yeast, animal and plant LPAATs (Lewin et al. 1999; Körbes et al. 2016) and
 241 have been shown to be the putative catalytic domain and binding domain, respectively. Proteins lacking
 242 either of these two motifs were discarded. These searches identified two putative LPAATs that we
 243 designated ptATS2a (protein ID: Phatr3_J11916) and ptATS2b (protein ID: Phatr3_J43099). The
 244 amino acid sequences of these two diatom LPAATs exhibit only 31%–38% identity to their homologs
 245 from bacteria, yeast, plant and green alga. Amino acid sequence alignment revealed that both LPAATs
 246 contain highly conserved NHX₄D domain, while the Thr residue in the EGTR motif was substituted by
 247 Met (EGMR) for ptATS2b (Fig. 1a).

248 To test the LPAAT activity of both ptATS2s, each gene was heterologously expressed in *E. coli*

249 *plsC* mutant SM2-1 that is unable to grow at 42°C (Coleman 1990). As shown on plate assay (Fig.1b),
 250 all the strains harboring an empty vector (pQE60) or a plasmid containing ptATS2a or ptATS2b
 251 exhibited normal cell growth at 30°C; although the *E. coli* mutant strain harboring pQE60 was
 252 nonviable at 42°C, heterologous expression of ptATS2a or ptATS2b fully restored cell growth at
 253 elevated temperature. To further determine the functionality of both ptATS2s, full-length coding region
 254 of ptATS2a or ptATS2b was heterologously expressed in Y02431 (*ale1* or *lca1Δ*), a yeast SLC4
 255 (Yor175c)-deficient mutant, under the control of the galactose-inducible *GALI* promoter. SLC4 acts as
 256 the major LPAAT in the yeast *S. cerevisiae*, and it is partially redundant with SLC1 in the synthesis of
 257 PA (Benghezal et al. 2007). Deletion of *SLC4* eliminated the microsomal biosynthesis of
 258 phosphatidylethanolamine (PE) and phosphatidylcholine (PC), and this mutant has also been shown to
 259 be hypersensitive to lyso-platelet-activating factor (lyso-PAF) because reacylation of lysoPAF that was
 260 required for cell viability was blocked (Chen et al. 2007). To examine whether heterologously
 261 expressed LPAAT can alleviate or eliminate the toxicity of lysoPAF on the Y02431 cells, we tested the
 262 growth of yeast transformants containing an empty vector or expressing ptATS2 on plates
 263 supplemented with different concentrations of lyso-PAF. As shown in Fig.1c, the mutant Y02431
 264 expressing ptATS2a or ptATS2b and harboring the empty pYES2/CT vector grew on plates containing
 265 10 µg/ml lyso-PAF. However, when plated with 30 µg/ml lyso-PAF, no growth for the control strain
 266 was observed while the mutant Y02431 expressing ptATS2a or ptATS2b was able to grow, confirming
 267 lysophospholipid acyltransferase activity of these two *P. tricornutum* LPAATs in the *S. cerevisiae*
 268 *lca1Δ* mutant.

269 **Both ptATS2a and ptATS2b encode a lysoPA acyltransferase**

270 The *S. cerevisiae slc4Δ* mutant (Y02431) showed a ~85% decrease in the total LPAAT activity of
 271 cellular homogenate relative to wild-type yeast, and the simultaneous deletion of *slc4* and *slc1* is lethal
 272 (Benghezal et al. 2007; Riekhof et al. 2007). We thus conducted an *in vitro* enzyme assay using the
 273 *slc4Δ* mutant (Y02431) to determine the specificity of both *P. tricornutum* LPAATs towards different
 274 lysophospholipids. The acyltransferase activity in microsomes from Y02431 cells, overexpressing
 275 ptATS2a or ptATS2b, was first measured using [¹⁴C]palmitoyl-CoA and *sn*-1-18:1-LysoPA as
 276 substrates. Of tested four colonies, at least three colonies of *S. cerevisiae* Y02431 expressing ptATS2a
 277 (Fig.2 a) or ptATS2b (Fig.2 b) had significantly higher LPAAT activity than that in control strain
 278 transformed with an empty vector, although there was a low level of endogenous LPAAT activity

279 encoded by SLC1 in Y02431. Plastidic LPAATs are likely to use acyl-acyl carrier protein (acyl-ACP)
280 in vivo, thus acyl-CoA-substrates might be not sufficient to detect LPAAT activities. Using
281 microsomal fractions of *S. cerevisiae* Y02431 expressing ptATS2a or ptATS2b as enzyme source and
282 [¹⁴C]16:0-CoA (Fig.2 c) or [¹⁴C]18:1-CoA (Fig.2 d) as acyl donor, we then examined their substrate
283 specificities towards other lysophospholipids. As shown in Fig.2 c,d, when *sn*-1-18:1-lysoPC, lysoPG,
284 lysoPS and lysoPE were used as acyl acceptor, respectively, the microsomal fraction from *S. cerevisiae*
285 Y02431 expressing ptATS2a or ptATS2b exhibited lower (15–80 times) acyltransferase activity than
286 with lysoPA. In the assay containing *sn*-1-18:1-lysoPE and [¹⁴C]16:0-CoA as substrates, the
287 microsomal fraction from *S. cerevisiae* Y02431 expressing ptATS2a had two-fold higher
288 acyltransferase activity than that from vector control (Fig.2c).

289 **Both ptATS2a and ptATS2b are localized in the chloroplast**

290 Prediction of transmembrane structures indicated that a transmembrane domain was located near
291 the N-terminus in the region of amino acids 95–123 for ptATS2a, whereas ptATS2b lacks a predicted
292 transmembrane region (Fig.S1). Prediction of putative N-terminal targeting signals revealed that the
293 presequence of ptATS2a contains a plastid-specific bipartite targeting signal (BTS), which is
294 characterized by the presence of an ASAFAP motif typical for proteins translocated across all four
295 membranes surrounding the plastids in diatoms (Apt et al., 2002; Kilian and Kroth, 2005; Gruber et al.,
296 2007). In contrast, only a classical signal peptide (amino acids 1–21) was predicted for ptATS2b. To
297 determine their subcellular localization, the expression of full-length ptATS2 fused with GFP was
298 visualized by confocal microscopy. The expression of ptATS2a-eGFP resulted in more or less
299 concentrated fluorescence signals directly surrounding the plastid autofluorescence, demonstrating a
300 similar fluorescence pattern to TPT4a and TPT4b, two putative triose phosphate translocators (Moog et
301 al. 2015). In contrast, ptATS2b was probably localized in the thylakoid according to the
302 ptATS2b-eGFP fluorescence signals present as spots in the plastid (Fig.3a,b), showing a similar
303 localization to the recently reported malonyl-CoA:ACP transacylase (Huang et al. 2023). To further
304 determine the localization and topologies of both ptATS2s in the plastid, we adapted a self-assembling
305 GFP approach (Cabantous et al. 2005). We fused last beta strand of GFP (GFP_S11), to ptATS2 and
306 co-transformed the diatom with strands 1-10 of GFP (GFP_S1-10) fused to topogenic signals either
307 directing the protein to the inner membrane space (IMS) or the stroma. As shown in Fig.3c,
308 fluorescence signals indicated a self-assembly of GFP in the stroma when co-expressing of

309 ptATS2a-GFP_S11 with stromal GFP_S1-10 but not in the IMS, indicating that ptATS2a is localized
 310 in the inner envelope membrane (IEM) with its C-terminus facing the plastid stroma. Whereas
 311 simultaneous expression of ptATS2b-GFP_S11 with the stromal marker fused to GFP_S1-10 resulted
 312 in GFP fluorescence, no signal was detectable in combination with the IMS marker (Fig.3d), indicating
 313 that ptATS2b is localized in the thylakoid with C-terminus facing the stroma.

314 **Generation of single *ptATS2* knockout mutants using multiplexed CRISPR/Cas9**

315 To characterize the functions of the two plastidial LPAATs in lipid synthesis in *P. tricornutum*, we
 316 generated single gene knockout mutants using multiplexed CRISPR/Cas9 method as described by
 317 Moosburner et al. (2020). We used Golden Gate cloning to create a set of constructs harboring
 318 multiplex gRNA expression cassettes for multiplexed genome editing. For each ptATS2 gene, we
 319 created two constructs each carrying three guide RNAs (gRNAs) separately expressed from
 320 independent siRNA promoter to simultaneously target endogenous loci in the *P. tricornutum* genome.
 321 These constructs carrying multiple gRNA expression cassettes, Cas9 expression cassette and zeocin
 322 resistance gene were introduced into *P. tricornutum* by bacterial conjugation. To examine mutation
 323 frequencies, the target sites/regions were PCR-amplified and subjected to DNA sequencing. After
 324 obtaining single colonies by several rounds of plating dilutions onto agar plates, we then designed a
 325 primer pair with one inside the deletion region and the other outside it, and performed PCR to screen
 326 positive colonies using genomic DNA as template (Fig.4a,b). The presence of a large fragment deletion
 327 within the predicted *PlsC* domain of *ptATS2a* and *ptATS2b* KO mutants was verified by the final DNA
 328 sequencing (Fig.4 c-f). These results show that creating deletions of large fragment with two or more
 329 gRNAs appears to be an efficient approach to achieve targeted mutagenesis. We chose two or three
 330 independent cell lines for each ptATS2 mutants to analyze lipid-associated phenotypes.

331

332 **Mutations of both *ptATS2* isoforms impair growth and cause strong reduction in TAG** 333 **accumulation**

334 The *ptATS2a* or *ptATS2b* mutants were first grown in static cultures, and their growth and N
 335 consumption of in nutrient-replete medium were very similar to that of wild type (WT) (Fig.5 a,b).
 336 Nonpolar lipid content was used as a readout for an impairment of whole glycerolipid homeostasis in *P.*
 337 *tricornutum*. It was evaluated by fluorescence intensity of algal cells stained with Nile Red, and
 338 verified by TAG measurement by TLC analyses. After 12 days of cultivation, nonpolar lipid and TAG

339 contents did not change in *ptATS2a*, whereas they were markedly reduced in *ptATS2b* compared with
 340 WT (Fig.5 c,d). When algal cultures were bubbled with air (low CO₂, c. 0.04% v/v CO₂), we
 341 observed that *ptATS2a* and *ptATS2b* showed a significantly compromised growth compared with
 342 WT (Fig.5 e). Nitrate concentrations in the medium in WT, *ptATS2a* and *ptATS2b* were decreased to
 343 the lowest level at day 7, 10 and 8 (Fig.5 f), respectively. Nonpolar lipid content and TAG
 344 accumulation were significantly reduced after 8 days of cultivation in both *ptATS2a* and *ptATS2b*
 345 compared with WT, and *ptATS2a* accumulated less TAGs than *ptATS2b* (Fig.5 g,h). To further
 346 examine the effect of *ptATS2* KO on lipid synthesis, we analyzed cell growth and lipid-associated
 347 phenotypes of mutants under N deprivation. There is no difference in cell density between the *ptATS2b*
 348 mutants and WT, but cell growth of *ptATS2a* was slower than WT after 2 days of N deprivation (Fig.5
 349 i). In addition, *ptATS2a* accumulated less nonpolar lipids and TAGs compared with WT during N
 350 deprivation (Fig.5 j,k). Although nonpolar lipid content in *ptATS2b* was reduced at day 2 and 3, it
 351 reached WT level at day 4; TAG accumulation in *ptATS2b* did not show marked reduction after 2 days
 352 of N deprivation (Fig.5 j,i). These results indicate that both *ptATS2s* mediate TAG synthesis but they
 353 play distinct roles under different conditions. We also measured the photosynthetic activities between
 354 WT and the mutants grown in the exponential phase. We observed an impairment of the photosynthetic
 355 capacity in the *ptATS2a* mutants as indicated by the significant decrease in PSII efficiency (F_v/F_m) and
 356 relative electron transport rate (rETR_{PSII}) when compared to WT, while there was no difference
 357 between WT and the *ptATS2b* mutants (Fig.S2).

358 **Impacts of the *ptATS2* mutation on glycerolipidomes**

359 Plastid-localized LPAATs could produce metabolic pools of PA feeding dedicated lipid
 360 biosynthetic pathways in the plastid and other cellular compartments. To examine the effect of *ptATS2*
 361 mutation on lipid composition and the distribution of different molecular species within each lipid class,
 362 we analyzed the glycerolipidomes of *P. tricornutum* WT and mutants grown in nutrient-replete
 363 medium for 4 d (+N) and in N-depleted medium for 4 d (-N). FA profiles were first analyzed for *P.*
 364 *tricornutum* WT and *ptATS2* mutants, revealing that the major FAs are palmitic acid (16:0), palmitoleic
 365 acid (16:1) and EPA at both conditions. KO of *ptATS2b* had little influence on FA composition at both
 366 +N and -N conditions whereas the mutation of *ptATS2a* significantly changed the proportion of three
 367 major FAs at N-depleted condition. Compared with WT, the main alterations of total FA profile in the
 368 *ptATS2a* mutants were observed at N-depleted condition by relative decreases in palmitic and

369 palmitoleic acids (from 34% in WT to 27% in *ptATS2a* of TFAs, and from 40% in WT to 34% in
 370 *ptATS2a* of TFAs, respectively), concomitant with an increase in the proportion of EPA (from 5% in
 371 WT to 8% in *ptATS2a* of TFAs) as well as 16:2, 16:3, 18:2 and 18:3 (Fig.S3). Detailed lipidomic
 372 analysis revealed that the most noticeable changes of TAGs and molecular composition in each lipid
 373 class were observed in the *ptATS2a* mutants. Almost no TAG synthesis was observed when WT and
 374 the mutants were grown in nutrient-replete medium for 4 d. However, after algal cells were grown in
 375 N-free medium for 4 days, N deprivation led to the accumulation of large amounts of TAGs, which
 376 accounted for 56% and 52% of total lipids in WT and *ptATS2b*, respectively, whereas the percentage of
 377 TAGs in total lipids in *ptATS2a* was declined to only 27% (Fig.6). In total lipids, SQDG, PG, PI and
 378 PC showed a decrease in the *ptATS2a* mutants in N-replete condition when compared to WT
 379 supporting a direct impact of the lack of *ptATS2a*. In the *ptATS2a* mutants the level of DAG, which is
 380 generated by removal of the *sn*-3 phosphate of PA, was decreased from 0.71% of total lipids in WT to
 381 0.51% ($P<0.01$) in N-replete condition, and from 2.43% of total lipids in WT to 1.24% ($P<0.0001$) in
 382 N-deprived condition (Fig.6).

383 The *ptATS2a* mutants also showed the most significant change of glycerolipid composition.
 384 Concerning plastid lipids supposed to be synthesized downstream of LPAATs, MGDG and DGDG in
 385 *ptATS2a* had a significant decrease in the levels of molecular species containing 20:5/16:3 and
 386 20:5/16:4 in both N-replete and N-deprived conditions (Fig.7a,b). Moreover, a significant decrease was
 387 also observed in *ptATS2b* in the levels of the 20:5/16:3 form representing the most abundant molecular
 388 species in MGDG and the 20:5/16:2 form in DGDG in N-replete condition. Compared to WT, two
 389 major SQDG species of 14:0/16:0 and 14:0/16:1 had a significant decrease in both *ptATS2a* and
 390 *ptATS2b* mutants, and the *ptATS2a* mutants also showed a decrease in the levels of 16:0/24:0 and
 391 16:1/16:0 in N-replete condition (Fig.7c). For PG, two major molecular species of 20:5/16:0 and
 392 20:5/16:1 showed a significant decrease in both conditions (Fig.7d). These changes suggest that
 393 inactivation of either *ptATS2a* or *ptATS2b* had a strong influence on molecular composition of all
 394 plastid glycerolipids.

395 PC, PE, and DGTA, likely synthesized in extra-plastidial membranes, also showed several
 396 noticeable alterations of molecular profiles in both *ptATS2* mutants. In PC, a significant decrease was
 397 observed in the *ptATS2a* mutants in the levels of those molecular species harbouring a 20:5 at the *sn*-1
 398 position and C16/C20/C22 FA at the *sn*-2 position in N-replete condition (Fig.8a, Supporting

399 information [Dataset S1](#)). In the *ptATS2a* mutants, two major PE species of 20:5/20:5 and 20:5/22:6
 400 showed a decrease in both N-replete and N-deprived conditions, whereas the *ptATS2b* mutants only had
 401 a slight decrease in the levels of PE 20:5/16:2 and 20:5/18:4 in N-replete condition ([Fig.8b](#)). In DGTA,
 402 a possible substrate for FA desaturation, the levels of at least 8 molecular species (14:0/16:1, 14:0/16:2,
 403 14:0/20:5, 16:1/18:3, 20:5/16:1, 20:5/22:5, 20:5/22:6 and 22:6/22:6) were significantly decreased in
 404 both *ptATS2a* and *ptATS2b* mutants in N-replete condition. In addition, the *ptATS2a* mutants also
 405 showed a decrease in the levels of 14:0/20:5, 16:1/18:3, 20:4/20:5, 20:5/20:5, 20:5/22:5 and 20:5/22:6
 406 in N-deprived condition ([Fig.8c](#)).

407 In the DAG pool, the most significant change was a decrease in the 14:0/16:1 form (46%,
 408 $P<0.0001$) and disappearance of 14:0/16:0 and 16:0/16:0 (non-detected) in the *ptATS2a* mutants in
 409 N-deprived condition (Supporting information [Dataset S1](#)). The decrease in 14:0/16:1 species in the
 410 DAG pool coincided well with the similar changes of this DAG backbone in MGDG, DGDG and
 411 SQDG ([Fig.7a-c](#)), which suggests that this DAG moiety of glycolipids might be of the same chloroplast
 412 origin. Further quantitative analysis of TAG molecular species revealed that in addition to the
 413 significant reduction of TAG content, TAGs from the *ptATS2a* mutants also showed altered molecular
 414 composition. Compared with WT, the major changes observed in *ptATS2a* were a decrease in the
 415 proportion of TAG species of 14:0/16:1/16:0, 16:0/16:0/16:0, 16:0/16:0/16:1, 16:1/16:1/16:0, and
 416 16:1/16:1/16:1 in both N-replete and N-deprived conditions ([Fig.9](#)). In contrast, almost no difference in
 417 TAG composition was observed between WT and the *ptATS2b* mutants. These results suggest that the
 418 activity of plastidial *ptATS2a* is involved, indirectly, in regulating TAG content as well as molecular
 419 composition of TAGs in *P. tricornutum*. Since plastid and extra-plastidial membrane lipids as well as
 420 cytosolic TAG are quantitatively and qualitatively impacted, these phenotypes reflect the importance of
 421 plastid LPAAT on the glycerolipid homeostasis at the whole cell level. Taken together, *ptATS2a*
 422 contributes to membrane lipid synthesis and to part of TAG accumulation under N deprivation, while
 423 the involvement of *ptATS2b* in these metabolic pathways, if any, was minor.

424 Discussion

425 In this study, we characterized two plastid-localized LPAATs from *P. tricornutum*, which are involved
 426 in the synthesis of almost all glycerolipids. Whereas *ptATS2a* is localized in the plastid IEM, the
 427 disruption of *ptATS2a* results in a decrease in the levels of SQDG and PG, which were recently verified
 428 to be synthesized in the plastid ([Huang et al. 2023](#)). Thus, PA as it is synthesized in the thylakoid by

429 *ptATS2a* could possibly be a precursor for SQDG and PG biosynthesis. Unexpectedly, disruption of
 430 *ptATS2a* led to an increase rather than a decrease in relative levels of MGDG and DGDG (Fig.6),
 431 which were also considered to be synthesized in the plastid. Possible causes are that there are multiple
 432 MGDG synthetic pathways present in *P. tricornutum* and the other pathways are upregulated in the
 433 *ptATS2a* mutants to compensate for the deficiency in thylakoid MGDG synthesis. Indeed, MGDG
 434 synthesis in *P. tricornutum* is catalyzed by three MGD isoforms, which localize to distinct subcellular
 435 compartments including thylakoid, periplastidal membrane, ER, and cytosol (our unpublished data),
 436 indicating these isoforms are likely non-redundant. These analyses suggest that MGDG, and its
 437 derivative DGDG, would not be restricted to thylakoid and IEM, and it is likely that there are multiple
 438 routes for MGDG synthesis in diatom cells. Ethanolamine-/choline-phosphotransferase involved in PE
 439 and PC synthesis was localized in the cER membrane (Huang et al. 2023), consistent with an
 440 occurrence of their syntheses in the cER. The *ptATS2a* mutants grown in N-replete condition showed a
 441 decrease in PC, suggesting that PA as it is synthesized at the IEM could possibly provide a direct
 442 substrate for PC synthesis. Although how PA/DAG produced by *ptATS2a* at IEM is transferred to the
 443 cER is not known, a PA transporter should be required for this process since PA itself cannot readily
 444 traverse the plastid membranes (OEM and PPM). However, we observed an increase in the level of PE
 445 in the *ptATS2a* mutants, indicating that PA produced by *ptATS2a* may not provide a precursor for PE
 446 synthesis in the cER and additional PE synthetic pathway possibly exists in *P. tricornutum*. For
 447 molecular composition of glycerolipids, our lipidomic analysis highlighted that chloroplast lipids in the
 448 *ptATS2a* mutants showed a decrease in the levels of 20:5/16:0 and 20:5/16:1 in PG, in the levels of
 449 20:5/16:3 and 20:5/16:4 in both MGDG and DGDG, and a moderate change in the profile of SQDG,
 450 besides a slight decrease in 20:5/16:0 molecular species (Fig.7). In addition, glycerolipids from
 451 endomembranes were also impacted, with a decrease of 20:5/(20:4, 20:5 and 22:6) in both PC and PE,
 452 and a decrease of 20:4/20:5, 20:5/(20:5, 22:5 and 22:6) in DGTA as well as 14:0/20:5 and 16:1/18:3 in
 453 this betaine lipid (Fig.8). Overall, the changes of lipid profiles were therefore consistent with a role of
 454 *ptATS2a* in the *sn*-2 acylation of *sn*-1-lysoPA substrates harboring a 20:4 or a 20:5 at the *sn*-1 position.

455 By contrast to *ptATS2a*, the impact of *ptATS2b* mutation on glycerolipid composition was less
 456 striking, with nearly no impact on PG, a moderate decrease of 20:5/(16:2 and 16:3) in MGDG, of
 457 20:5/(16:1, 16:2 and 16:3) in DGDG, of 20:5/16:0 in SQDG, of 20:5/(20:4, 20:5 and 22:6) in PC and of
 458 20:4/20:5 and 20:5/20:5 in DGTA. For this isoform, the changes of lipid profiles were also consistent

459 with a role of ptATS2b in the *sn*-2 acylation of a *sn*-1-acyl-glycerol-3-P substrate harboring a 20:4 or a
460 20:5 at the *sn*-1 position (Fig.10). Based on the molecular species increasing in *ptATS2b*, some of them
461 correspond to molecular species shown to decrease in the *ptATS2a* mutants, such as 20:5/16:0 in
462 DGDG and PG, and 20:4/20:5 and 20:5/20:5 in DGTA in N-replete condition, suggesting that ptATS2a
463 was able, at least partly, to compensate the *ptATS2b* mutation. This compensation mechanism
464 occurring mainly in the plastid was consistent with the absence of impact on the profile of cytosolic
465 TAG.

466 Our finding that loss-of-function mutations in single *ptATS2* do not completely abolish plastid lipid
467 synthesis and TAG accumulation also implies that there is partially functional redundancy between
468 both ptATS2s and possibly non-plastidial LPAAT isoforms not considered in the present study.
469 Furthermore, our complementation experiment showed that overexpression of ptATS2b in the *ptATS2a*
470 mutants could partly restore algal cell growth and TAG-associated phenotype and *vice versa* (Fig.S4),
471 supporting that disruption of a plastidial ptATS2 gene could, at least in part, be biochemically and
472 physiologically compensated by the other plastid-located ptATS2.

473 Quantitative RT-PCR analyses showed that transcript levels of both *ptATS2a* and *ptATS2b* increased
474 during N deprivation for 3 d (Fig.S5), indicating an important role of these two plastidial LPAATs in N
475 starvation-induced TAG accumulation. Consistent with their gene expression patterns, disruption of
476 either *ptATS2* resulted in a significant decrease in the amounts of TAGs during N deprivation for 3 d
477 (Fig.5j,k). Interestingly, a greater extent of decrease in the amount of TAGs was seen in *ptATS2a* than
478 that in *ptATS2b* at the stationary phase and under N-deprived condition when compared with WT
479 (Fig.5h,k,i), which is in agreement with the greater extent of increase in the transcript levels of
480 *ptATS2a* compared to that of *ptATS2b*. These results could indicate a greater contribution of ptATS2a
481 than that of ptATS2b to TAG synthesis under N-starved condition. However, previous study showed
482 that overexpression of ptATS2b increased TAG content by 1.8-fold with a significant increase in
483 PUFAs, indicating its potential regulatory role in TAG synthesis (Balamurugan et al. 2017; Wang et al.
484 2018). In the green alga *Chlamydomonas*, RNAi-mediated suppression of *CrLPAAT2*, which encodes
485 an ER-located chlorophyte-specific LPAAT, caused a reduction of TAG content under N deprivation
486 (Kim et al. 2018). However, the abundance of the major chloroplast membrane glycerolipids including
487 MGDG, DGDG and SQDG was not affected in these *Chlamydomonas* RNAi strains. In contrast to the
488 negligible role of CrLPAAT2 under nutrient-replete conditions in *Chlamydomonas*, our results

489 demonstrate that both plastidial LPAATs are required for the biosynthesis of storage TAGs and almost
 490 all chloroplast and extra-plastidic membrane glycerolipids in nutrient-replete and N-starved conditions.
 491 The most striking effect of disruption of *ptATS2* on lipid metabolism was the dramatic decrease in the
 492 content of storage TAGs, which could be explained by two possible causes: (1) the reduced supply of
 493 plastid-generated PA/DAG that would be further exported to the cytosol as the glycerol backbone for
 494 ER-associated TAG synthesis; (2) inhibition of plastidial *de novo* synthesis of FAs. The involvement of
 495 a route from chloroplast glycerolipids to TAG could be supported by the abundant presence of
 496 chloroplast glycerolipid C16 FAs in TAG and the concomitant reduction of some DAG molecular
 497 species in chloroplast galactoglycerolipids and TAGs in the *ptATS2a* mutants. This is also consistent
 498 with recent cell biochemistry and proteomic studies of lipid droplets indicating a tight apposition of this
 499 cytosolic TAG storage body with the surface of the plastid (Lupette et al. 2019; Leyland et al. 2020).

500 In conclusion, our data outline the crucial role of two plastidial LPAATs for the biosynthesis of
 501 chloroplast thylakoid membrane lipids, extra-plastidic membrane glycerolipids and storage TAGs in *P.*
 502 *tricornutum*. We found that a single plastidial LPAAT enzyme can regulate different downstream
 503 glycerolipid pathways under different conditions. The plastid pathway of thylakoid lipid biosynthesis
 504 was strongly impaired in either *ptATS2a* or *ptATS2b* mutants. Our data highlight an important role of
 505 20:5-lyso-PA, as a candidate lipid intermediate in the import of 20:5 from the cytosol to the plastid *via*
 506 the so-called ‘omega pathway’. In addition, the mutants also showed compromised cell growth and
 507 strikingly reduced TAG accumulation when algal cultures were bubbled with air. Therefore, it seems
 508 likely that both *ptATS2s* play an essential role in algal cell growth consistent with their involvement in
 509 the generation of PA, a central metabolite of primary lipid metabolism and a regulatory molecule in the
 510 chloroplast. Future work on the detailed characterization of the plastidial *ATS1* and additional
 511 ER-located LPAATs will extend our appreciation of the individual contribution of LPAATs to lipid
 512 metabolism in this EPA-rich oleaginous diatom.

513

514 **Figure legends**

515 **Fig.1.** Identification of two plastid LPAAT acyltransferases in *P. tricornutum*. (a) Alignment of *P.*
 516 *tricornutum* plastidic LPAATs and their homologs from plants, green algae, bacteria and yeast.
 517 Conserved residues are in white font on a red background and semi-conserved residues are in
 518 black font on a yellow background. The amino acid sequences of *ptATS2a* (Phatr3_J11916) and

519 ptATS2b (Phatr3_J43099) were aligned together with those of characterized LPAATs from
 520 *Arabidopsis thaliana* (AtATS2), *Brassica napus* (BnATS2), *Chlamydomonas reinhardtii*
 521 (CrLPAAT1), *Escherichia coli* (EcPlsC) and *Saccharomyces cerevisiae* (ScSLC1). (b) Expression
 522 of either ptATS2a or ptATS2b could rescue the high temperature sensitivity of the *E. coli plsC*
 523 mutant, which is deficient in LPAAT. (c) Lyso-PAF (lyso-platelet-activating factor) sensitivity
 524 test of the *S. cerevisiae lca1Δ* mutant harboring empty vector (pYES2/CT) or expressing either
 525 ptATS2a or ptATS2b. Yeast cells were grown in SC-Ura medium supplemented with 2% glucose
 526 overnight at 28°C with shaking then transferred to induction medium for 12 h to induce protein
 527 expression. Cultures were serial diluted from OD₆₀₀=2.0 to 0.1. A measure of 2 μl from each
 528 dilution was spotted on SC-Ura plates containing 10 or 30 μg/ml lyso-PAF, respectively. The
 529 plates were incubated at 28°C for 2 days.

530

531 **Fig.2.** Acyltransferase activity of ptATS2a and ptATS2b towards different lysophospholipids. The
 532 enzyme assays were performed with microsomal preparations from the yeast *slc4Δ* mutant
 533 expressing the ptATS2 gene or harboring empty vector (pYES2/CT) with different
 534 lysophospholipids as acyl acceptor and [¹⁴C]16:0-CoA or [¹⁴C]18:1-CoA as acyl donor. The
 535 LPAAT activities of ptATS2a (a) and ptATS2b (b) were measured using *sn*-1-C18:1-lysoPA and
 536 [¹⁴C]16:0-CoA as substrates. The amounts of synthesized [¹⁴C]PA during reaction time were
 537 treated as the result of LPAAT activity. The lysophospholipid acyltransferase activities of
 538 ptATS2a and ptATS2b were tested using [¹⁴C]16:0-CoA (c) or [¹⁴C]18:1-CoA (d) as acyl donor.
 539 In the enzyme assay, four different lysophospholipids were used as acyl acceptors:

540 *sn*-1-C18:1-lysoPE, *sn*-1-C18:1-lysoPC, *sn*-1-C18:1-lysoPG, or *sn*-1-C18:1-lysoPS.

541 Acyltransferase activities were calculated based on the amounts of synthesized [¹⁴C]
 542 phospholipids during the reaction time. The representative results of three independent yeast
 543 transformants were shown. Mean values and standard deviation are presented (n≥3). Asterisk
 544 indicates significant difference from the empty vector control as determined by the Student's *t*-test
 545 (*, *P*<0.05). PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol;
 546 PS, phosphatidylserine.

547

548 **Fig.3.** Plastid localization of both ptATS2s. (a, b) ptATS2a and ptATS2b fused to enhanced GFP

549 were transfected into *P. tricornutum* to determine subcellular localization. (c, d) the
 550 self-assembling split GFP system was adapted in *P. tricornutum*. Both ptATS2a and ptATS2b,
 551 fused to GFP_S11 did not show a fluorescence signal when co-expressed with the IMS marker
 552 MGD1 fused to GFP_S1-10, whereas a clear GFP signal could be observed upon co-expression
 553 with the stromal-targeted AtpC_BTS-GFP_S1-10, respectively. TL, transmitted light; PAF, plastid
 554 autofluorescence; GFP, enhanced green fluorescence protein; PAF/GFP, overlay of plastid and
 555 GFP fluorescence. PAF is shown in red and GFP fluorescence in green. BTS, bipartite targeting
 556 signal; PPC, periplastidal compartment; IMS, intermembrane space; Str, plastid stroma; Scale bar
 557 represents 5 or 8 μm .

558

559 **Fig.4.** Genome editing of *ptATS2a* and *ptATS2b* using multiplex CRISPR/Cas9 method led to the
 560 generation of fragmental deletions in *P. tricornutum*. Schematic diagrams show the fragmental
 561 deletions between two targeted sites of the *ptATS2a* (a) and *ptATS2b* (b) mutants. A pair of
 562 primers was designed to detect the large fragmental deletions induced by multiplex CRISPR/Cas9
 563 in the *ptATS2a* (c) and *ptATS2b* (d) mutants with PCR using genomic DNAs and total
 564 complementary DNA (cDNA) as template. For DNA sequencing, PCR was carried out using
 565 primer pairs flanking the predicted target sites, and the PCR products were sequenced directly
 566 using internal specific primers. Representative sequences of fragment deletions in the *ptATS2a* (e)
 567 and *ptATS2b* (f) mutants were aligned with that of wild type. The PAM region is shown in blue
 568 color letters, and the number at the end shown in red color letters indicates deleted (-) bases
 569 between two Cas9 cut sites.

570

571 **Fig.5.** Phenotype analyses of the *ptATS2a* and *ptATS2b* mutants. (a) Growth curves of the
 572 *ptATS2a* and *ptATS2b* mutants incubated in static cultures for 18 days. Cell densities were
 573 determined with a Z2 Coulter Counter. Results are the average of three biological replicates
 574 (independent cell lines) with error bars indicating standard deviations ($n=3$). (b) Nitrate
 575 concentrations in culture media for the *ptATS2a* and *ptATS2b* mutants incubated in static cultures.
 576 (c) Nile red fluorescence intensities of *ptATS2a* and *ptATS2b* mutant cells incubated in static
 577 cultures for 18 days. Statistical analysis using paired-sample Student's *t* test was performed by
 578 GraphPad Prism (v9.0): **, $P<0.01$; ***, $P<0.001$. (d) A thin-layer chromatogram of total lipids

579 from wild type, the *ptATS2a* and *ptATS2b* mutants incubated in static cultures for 13 d, 14 d, and
 580 15 d. Each lipid sample was extracted from $\sim 5 \times 10^7$ cells. TAGs were visualized with 0.01% (w/v)
 581 primuline reagent. (e) Growth curves of the *ptATS2a* and *ptATS2b* mutants under air bubbling
 582 condition for 12 days. Data are the average of three biological replicates (independent cell lines)
 583 with error bars indicating standard deviations ($n=3$). (f) Nitrate concentrations in culture media for
 584 the *ptATS2a* and *ptATS2b* mutant cultures bubbled with filtered air. (g) Nile red fluorescence
 585 intensities of the *ptATS2a* and *ptATS2b* mutant cultures bubbled with filtered air for 6-12 days.
 586 Statistical analysis using paired-sample Student's *t* test was performed by GraphPad Prism (v9.0):
 587 **, $P < 0.01$; ***, $P < 0.001$. (h) TLC of total lipids from wild type and the *ptATS2a* and *ptATS2b*
 588 mutants grown in air-bubbling condition for 8 d, 9 d and 10 d. Each lipid sample was extracted
 589 from $\sim 5 \times 10^7$ cells. (i) Growth curves of the *ptATS2a* and *ptATS2b* mutants under nitrogen
 590 deprivation for 4 days. Data are the average of three biological replicates (independent cell lines)
 591 with error bars indicating standard deviations ($n=3$). (j) Nile red fluorescence intensities of algal
 592 cells of the *ptATS2a* and *ptATS2b* mutants grown under nitrogen deprivation for 4 days. Statistical
 593 analysis using paired-sample Student's *t* test was performed by GraphPad Prism (v9.0): **,
 594 $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. A TLC of total lipids from wild type and the *ptATS2a* (k)
 595 and *ptATS2b* (l) mutants grown under nitrogen deprivation for 4 d verified the change of TAG
 596 accumulation. Each lipid sample was extracted from $\sim 5 \times 10^7$ cells.

597

598 **Fig.6.** Quantification of different molecular species of TAG in the *ptATS2a* knockdown (KD) and
 599 *ptATS2b* knockout mutants in three biological replicates using LC-MS. Each TAG species is
 600 shown as N:M, where N is the number of carbon atoms in acyl-chains and M is the number of
 601 C=C double bonds. The size of the corresponding circle followed by each TAG species indicates
 602 the average percentage of total TAG represented by wild type. A heat map shows the percentage
 603 abundance of each species in each biological replicates relative to the average percentage
 604 abundance of that species in wild type.

605

606 **Fig.7.** Impact of *ptATS2a* knock down (KD) and *ptATS2b* knockout (KO) on the relative amounts
 607 (mol%) of molecular species in plastidial glycolipids including (a)
 608 monogalactosyldiacylglycerol/MGDG, (b) digalactosyldiacylglycerol/DGDG and (c)

609 sulfoquinovosyldiacylglycerol/SQDG. *P. tricornutum* wild type and mutant cells were harvested
 610 at day 8 and day 10 from algal cultures bubbled with filtered air. Data are means \pm SD ($n=3$) and
 611 asterisks indicate significant difference from wild type as determined by the Student's *t*-test (*,
 612 $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

613

614 **Fig.8.** Impact of ptATS2a knockdown (KD) and ptATS2b knockout (KO) on the relative amounts
 615 (mol%) of molecular species in (a) phosphatidylcholine (PC) and (b) the betaine glycerolipid
 616 diacylglycerol-hydroxymethyl-*N,N,N*-trimethyl- β -alanine (DGTA). *P. tricornutum* wild type and
 617 mutant cells were harvested at day 8 and day 10 from algal cultures bubbled with filtered air. Data
 618 are means \pm SD ($n=3$) and asterisks indicate significant difference from wild type as determined
 619 by the Student's *t*-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

620

621 **Fig.9.** Impact of ptATS2a knock down (KD) and ptATS2b knockout (KO) on the relative amounts
 622 (mol%) of molecular species in plastidial glycolipids from *P. tricornutum* wild type and the
 623 mutants grown under nitrogen deprivation for 4 days. The glycolipids analyzed include (a)
 624 monogalactosyldiacylglycerol/MGDG, (b) digalactosyldiacylglycerol/DGDG, (c)
 625 sulfoquinovosyldiacylglycerol/SQDG, and (d) phosphatidylglycerol/PG. Data are means \pm SD
 626 ($n=3$) and asterisks indicate significant difference from wild type as determined by the Student's
 627 *t*-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

628

629 **Fig.10.** A proposed model depicting the role of ptATS2a and ptATS2b in the biosynthesis of
 630 chloroplast membrane lipids and TAGs in the diatom *P. tricornutum*. This model summarized the
 631 major steps of the generation of PA/DAG in the plastid and the ER, providing precursors of
 632 glycerol backbone for the synthesis of chloroplast membrane lipids and storage TAGs,
 633 respectively. The plastid pathway begins with acylation of G3P with acyl-ACP by ATS1, leading
 634 to the generation of lysoPA. Further acylation of the *sn*-2 position of lysoPA to form PA is
 635 catalyzed by the chloroplast-localized ATS2. PA is dephosphorylated to form DAG, which is used
 636 for biosynthesis of chloroplast membrane glycolipids including MGDG, DGDG and SQDG. The
 637 protein localization and alteration of glycerolipidomic profiles of the *ptATS2a* and *ptATS2b*
 638 mutants support their role in the chloroplast in the *sn*-2 acylation of

639 *sn*-1-acyl-glycerol-3-phosphate substrates harboring a 20:5 at the *sn*-1 position. The Kennedy
640 pathway for TAG assembly is also shown to illustrate the contrasting reaction of storage TAG
641 accumulation under nitrogen starvation. In this pathway, ER-located lysophosphatidic acid
642 acyltransferase (LPAAT) catalyzes the conversion of lysoPA to PA, which is then
643 dephosphorylated by phosphatidic acid phosphatase (PAP) to form DAG. As an immediate
644 precursor, DAG is used for TAG production under nitrogen limitation through the
645 acyl-CoA-dependent acylation of the *sn*-3 position by diacylglycerol acyltransferase. GPAT,
646 glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; DGAT,
647 diacylglycerol acyltransferase; LACS, long-chain acyl-CoA synthetase; G3P, lysoPA,
648 lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; MGDG,
649 monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

650

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666

667 **Authors' contributions**

668 LY, AP, KJG, FR, XH and HJ performed experiments. JJ, EM, AB and HH analyzed the data. A.
 669 Allen contributed to the multiplexed CRISPR/Cas9 vectors and methods. YG and A. Amato
 670 planed and designed the research. YG wrote the manuscript and JJ, EM and A. Amato revised the
 671 manuscript.

672 **Data availability**

673 The data that support the findings of this study are available in the supplementary material of this
 674 article.

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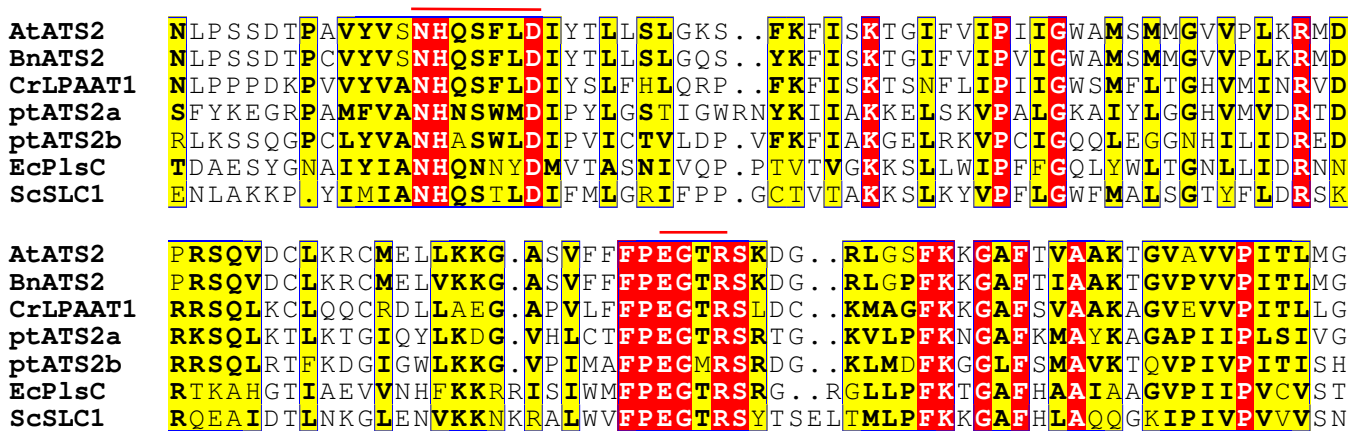
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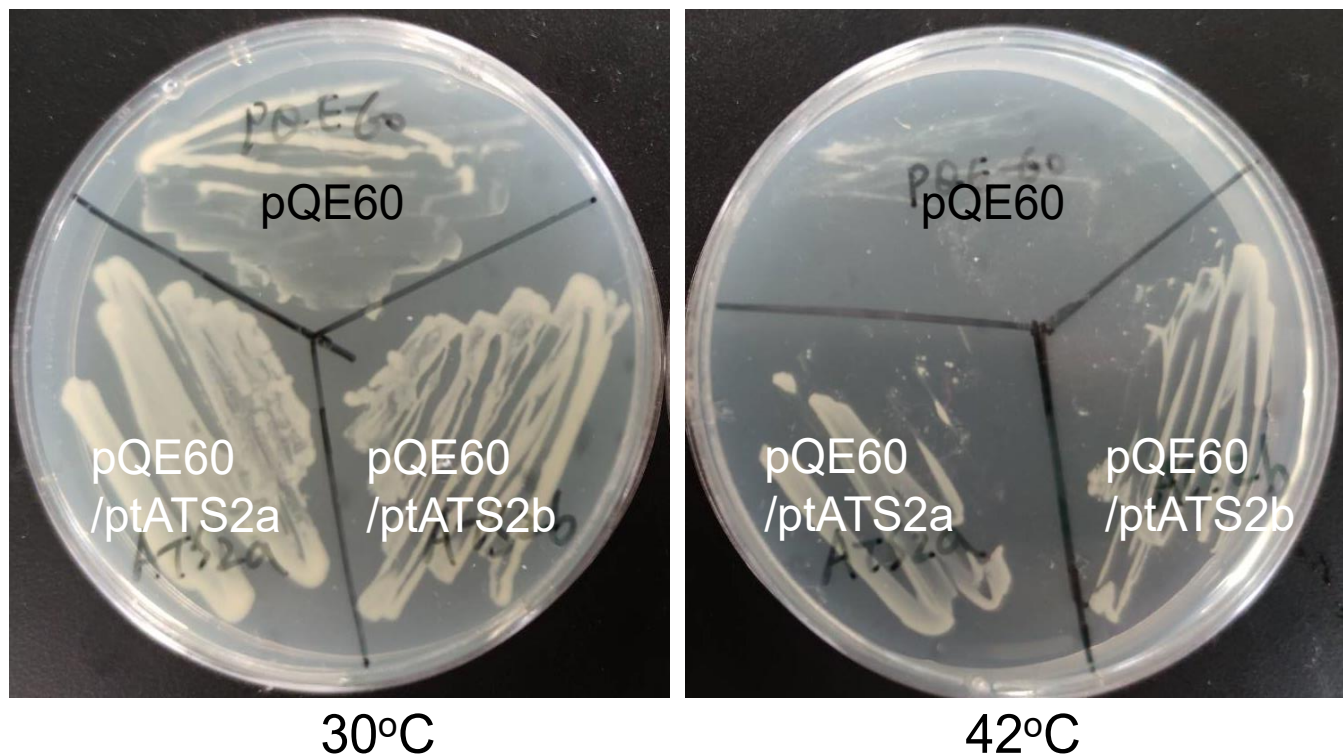
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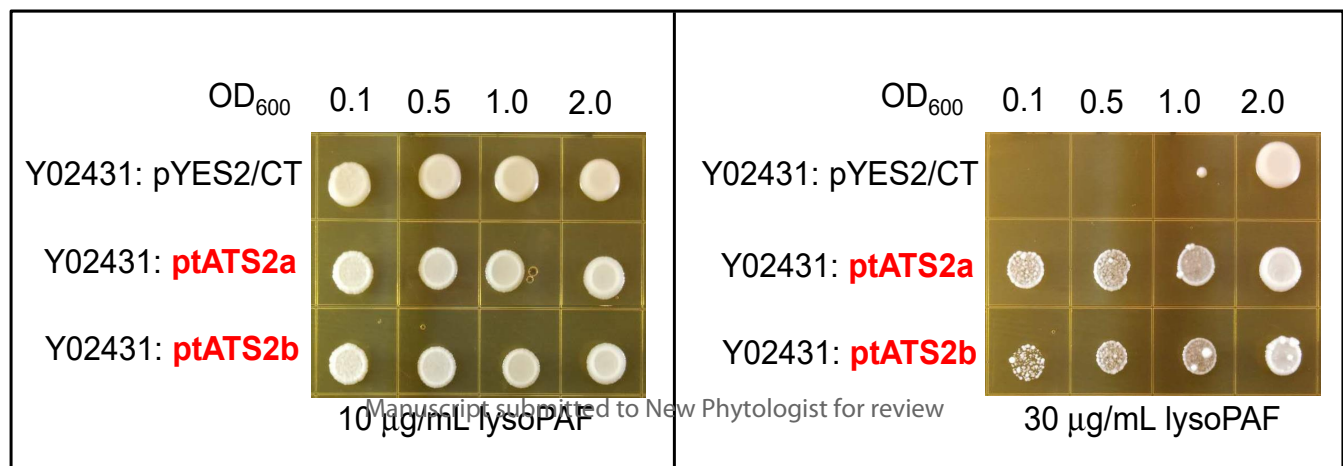
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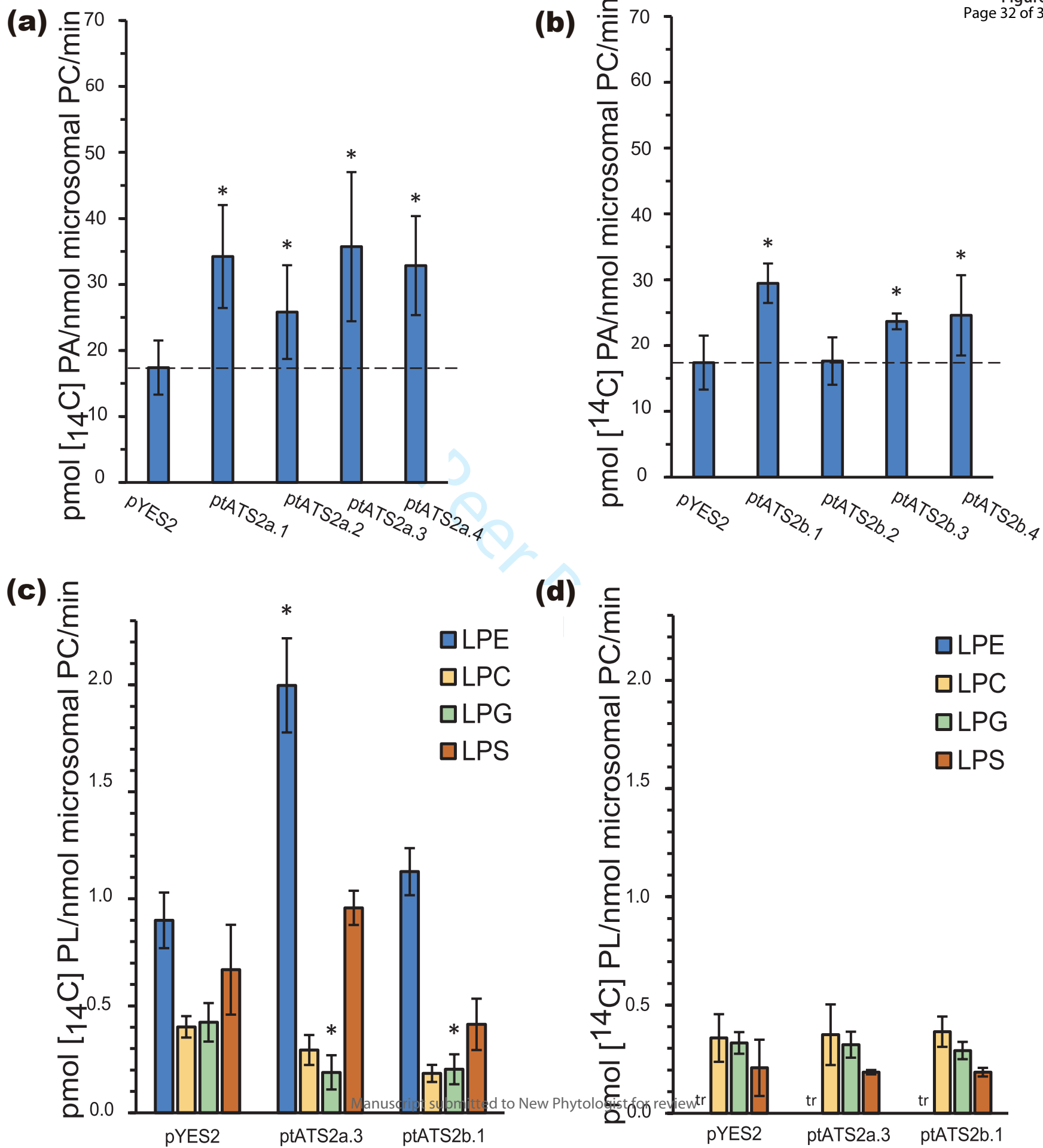


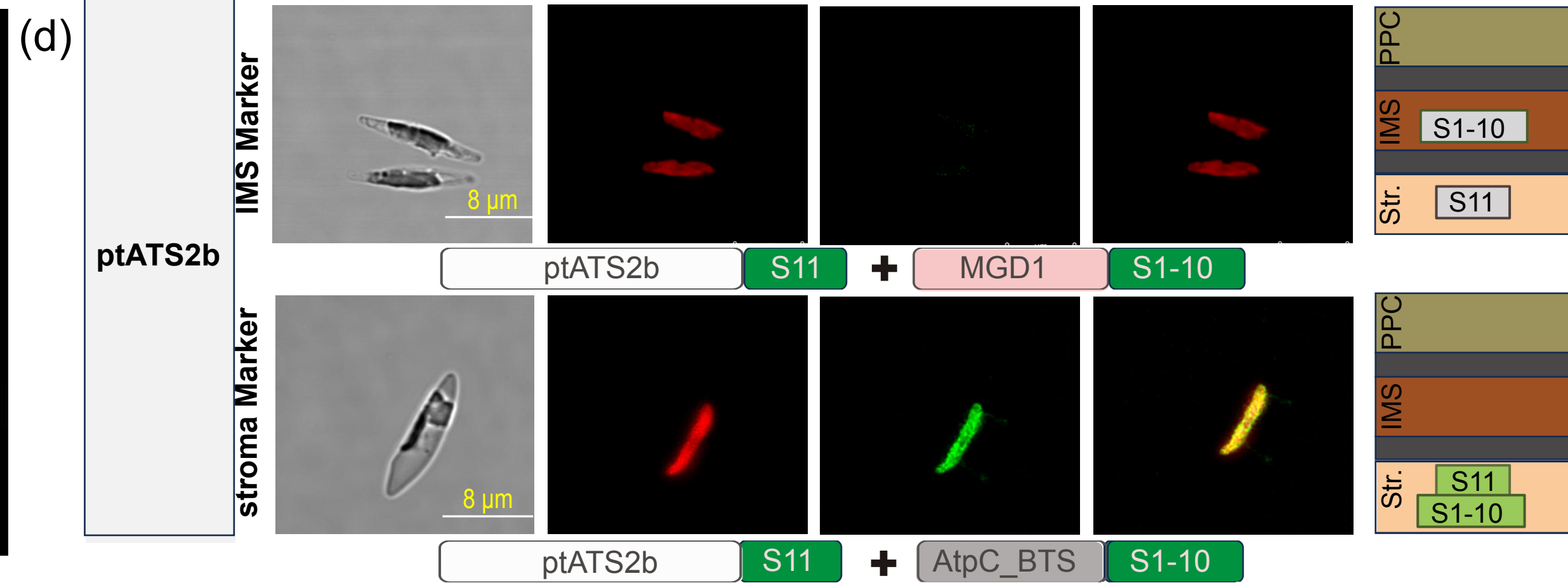
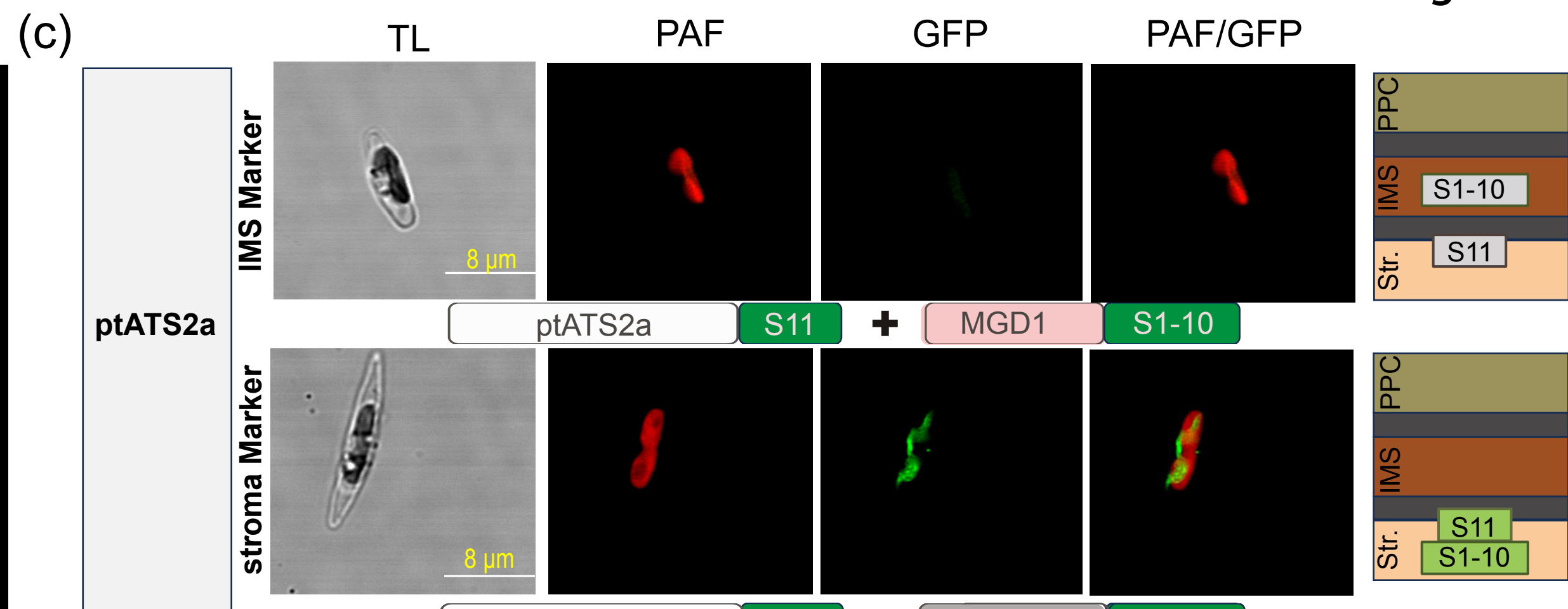
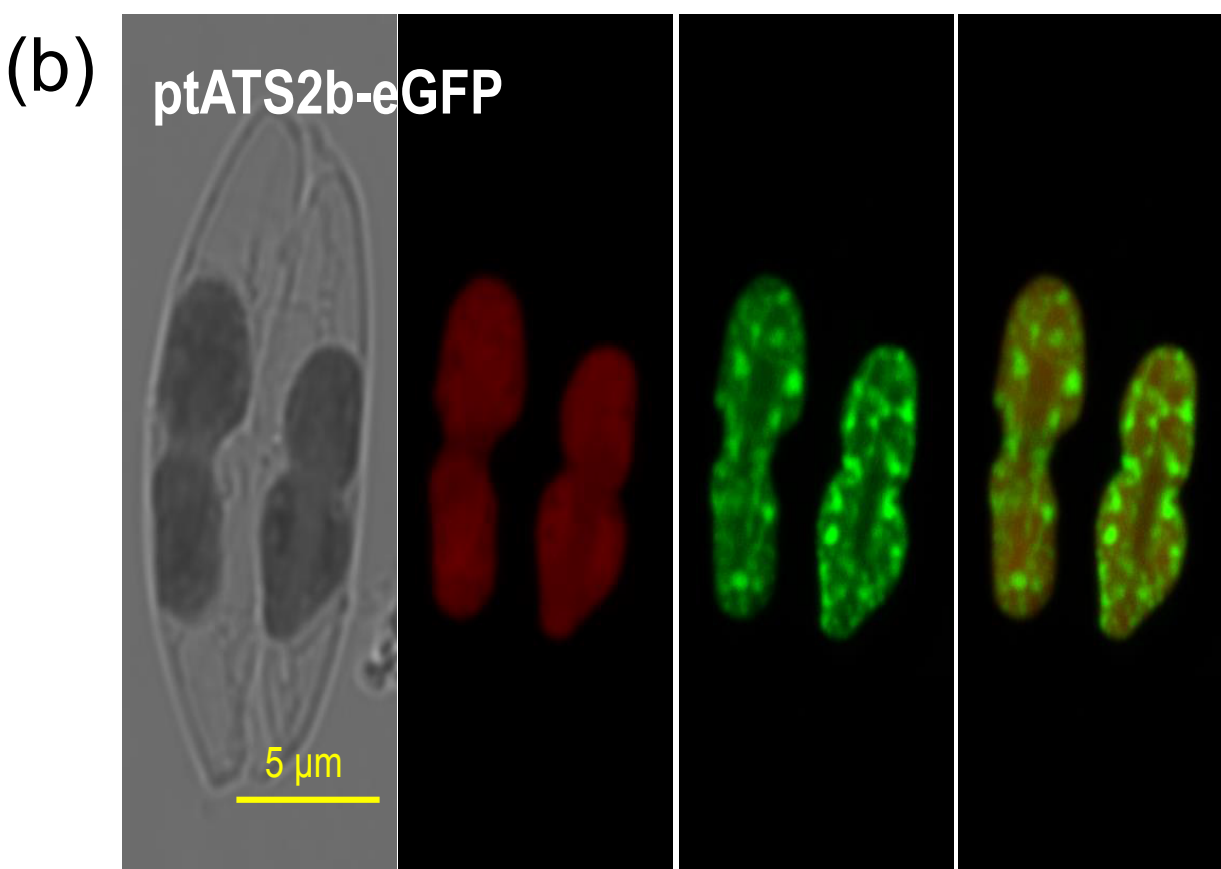
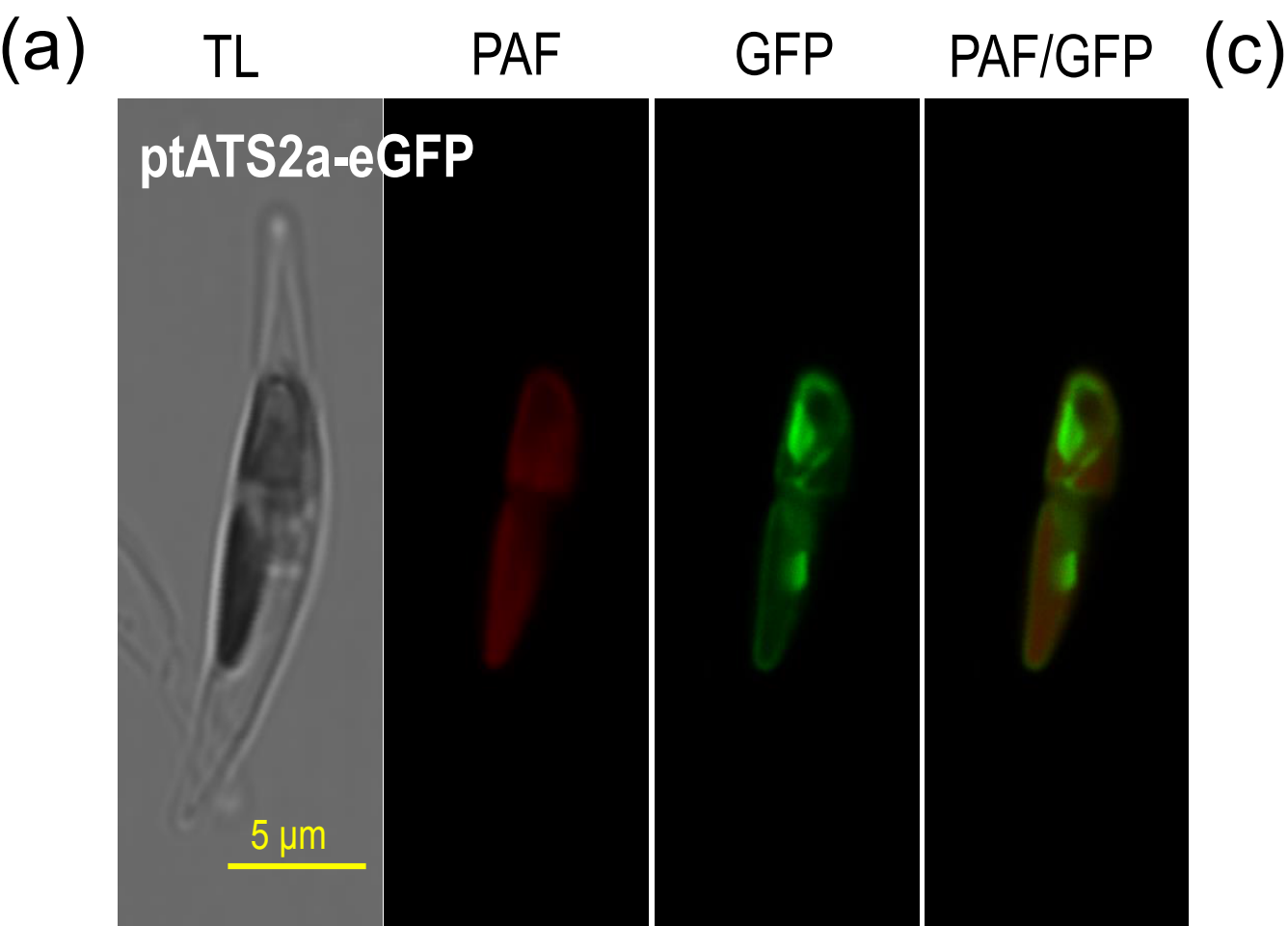
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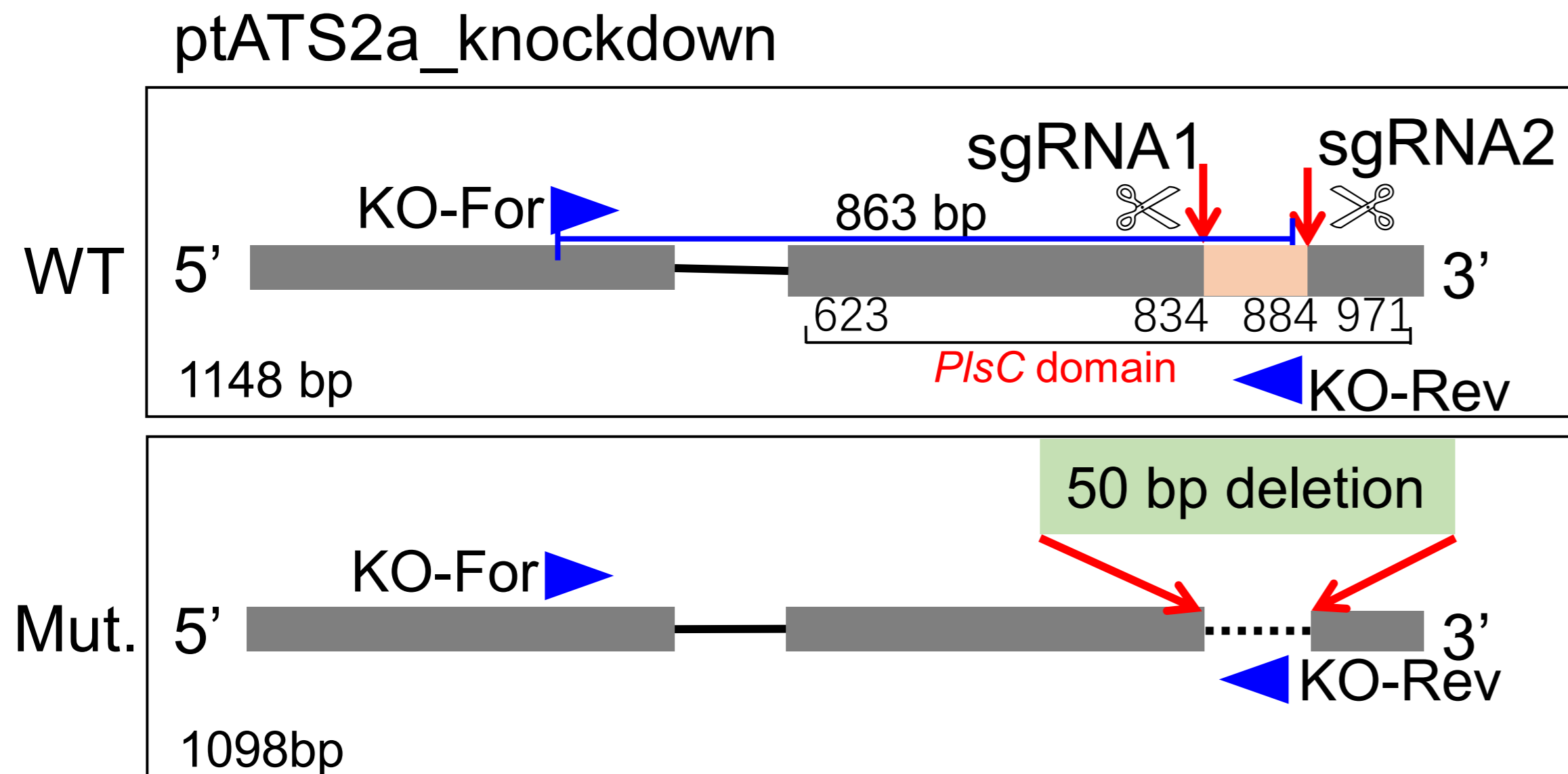
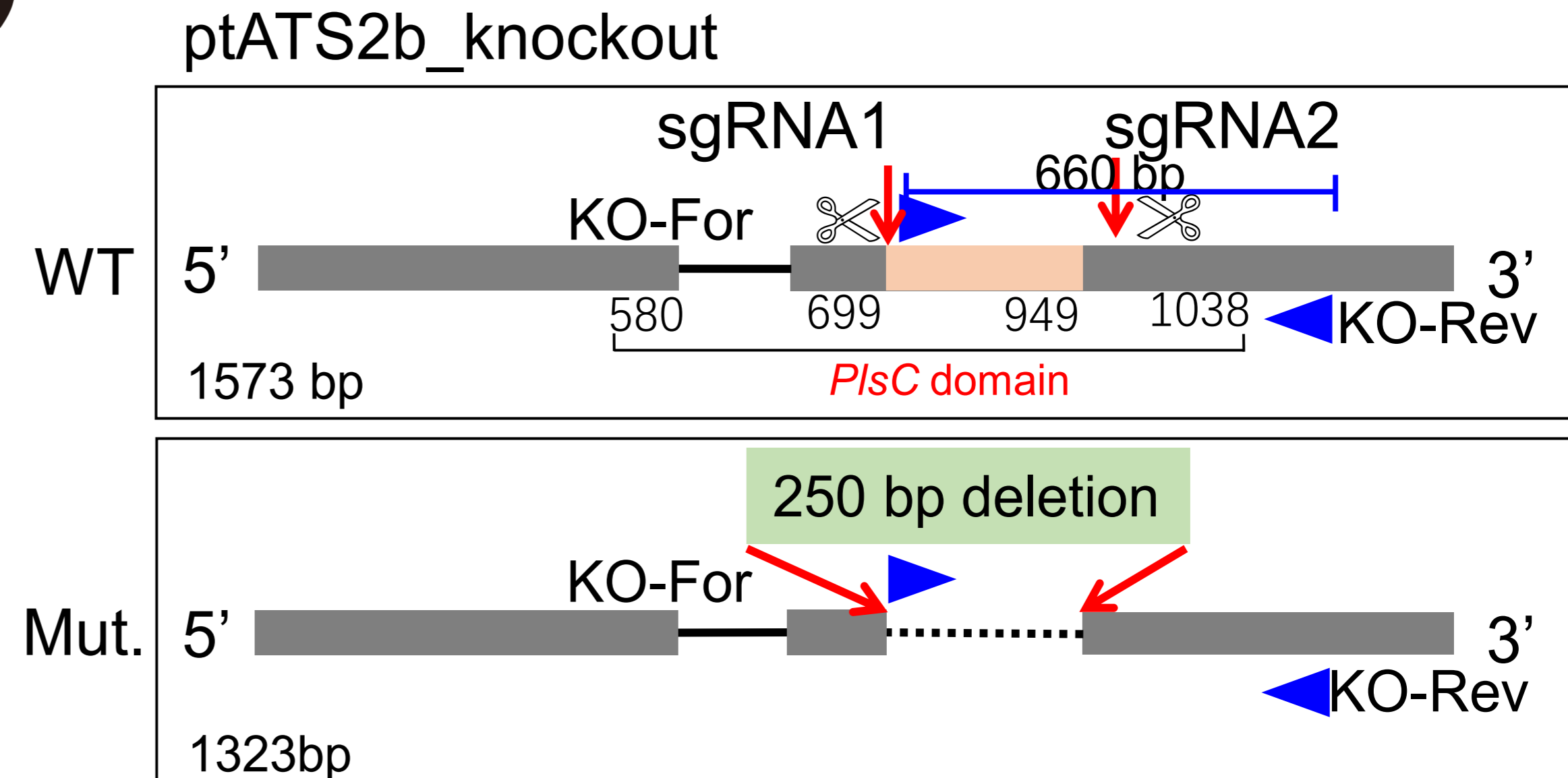
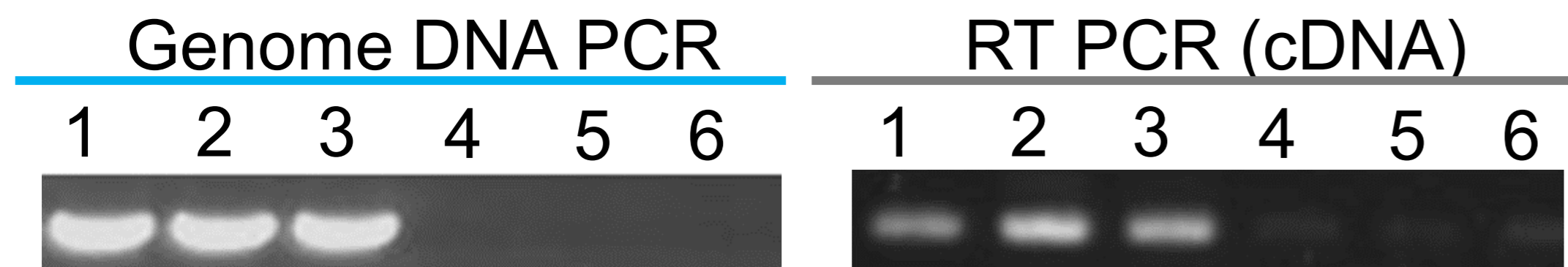
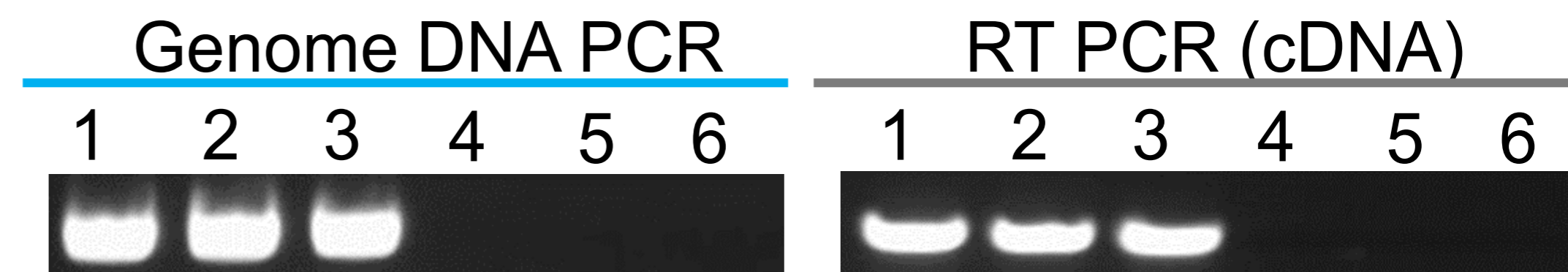


(c)





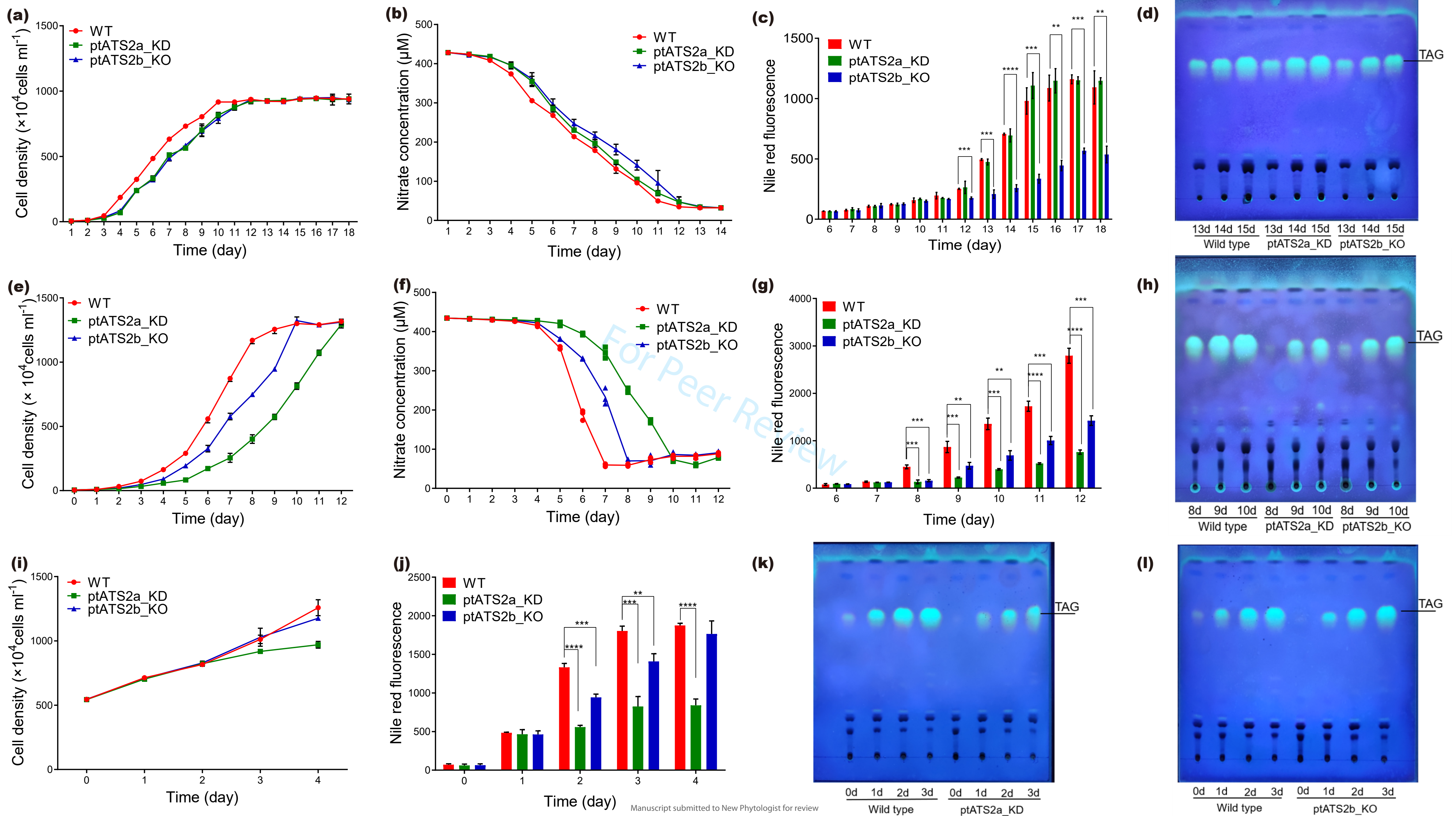


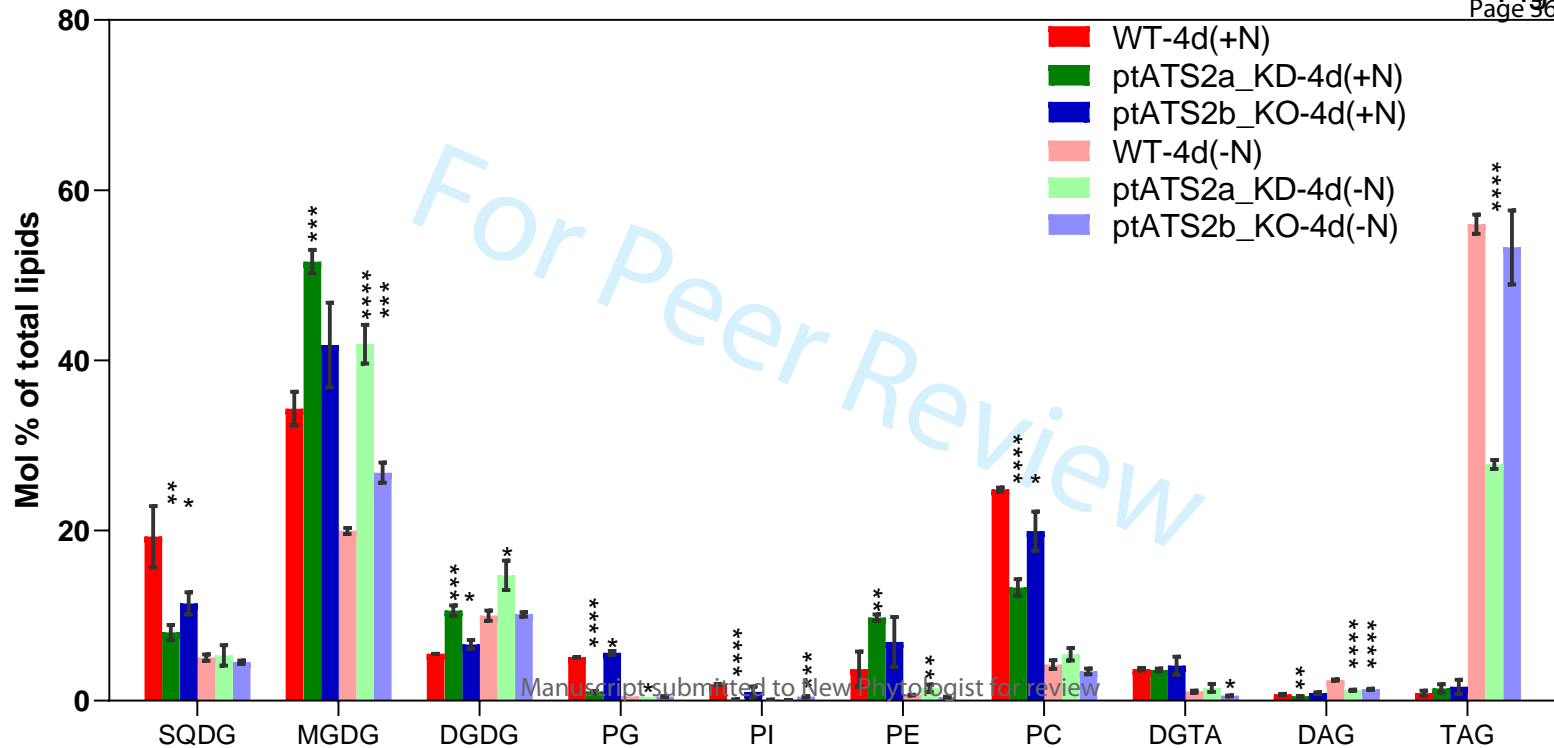
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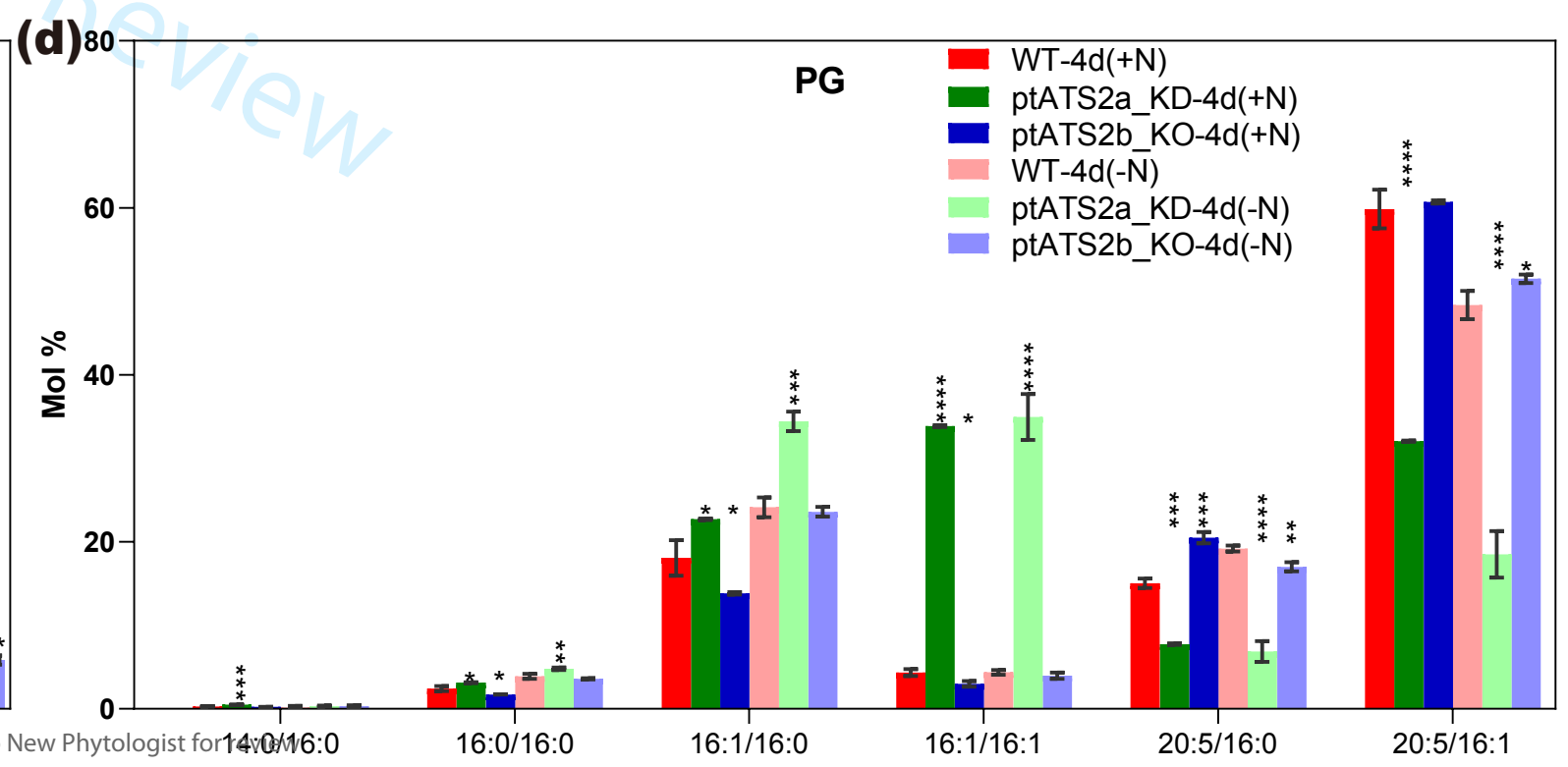
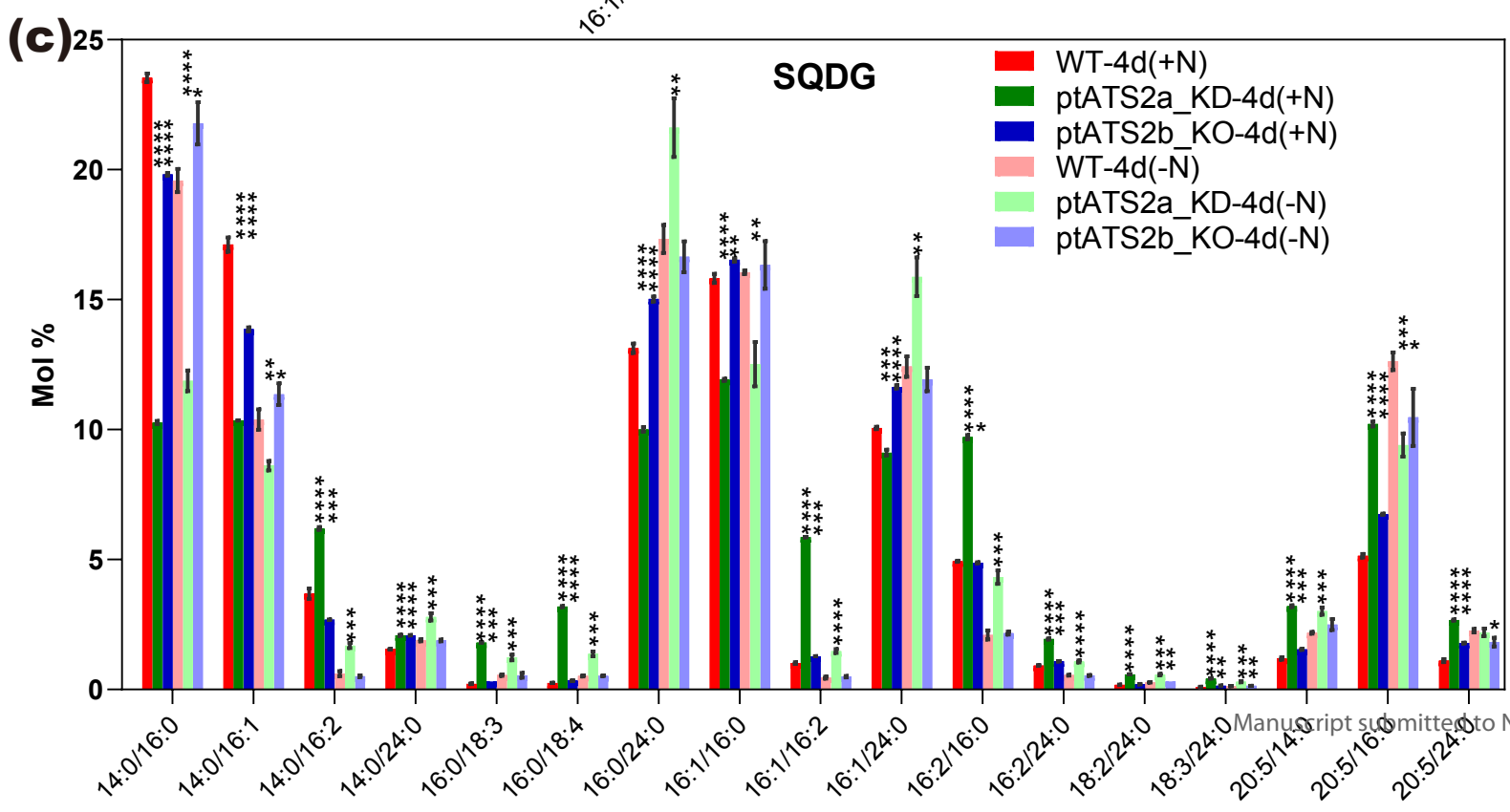
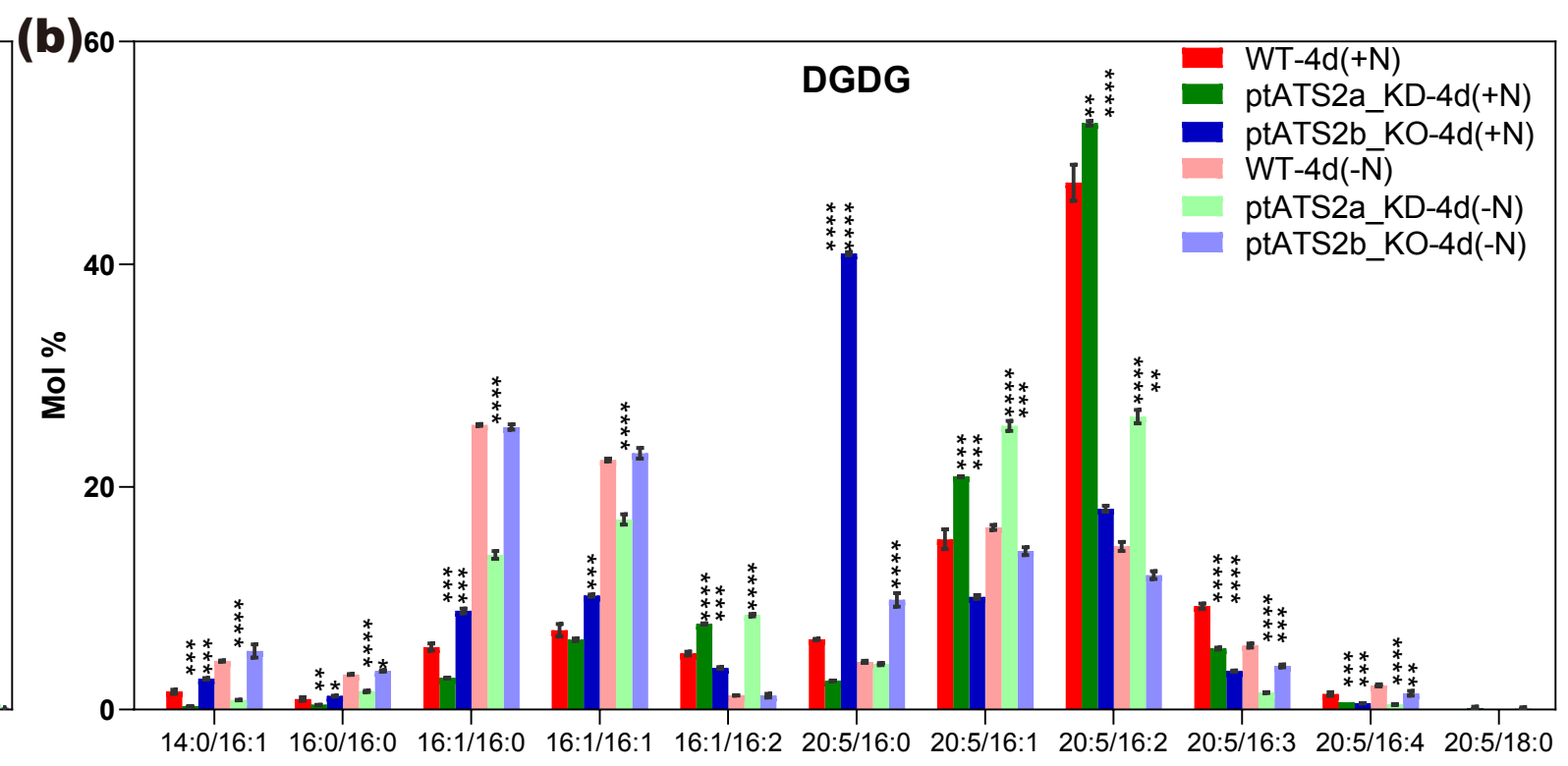
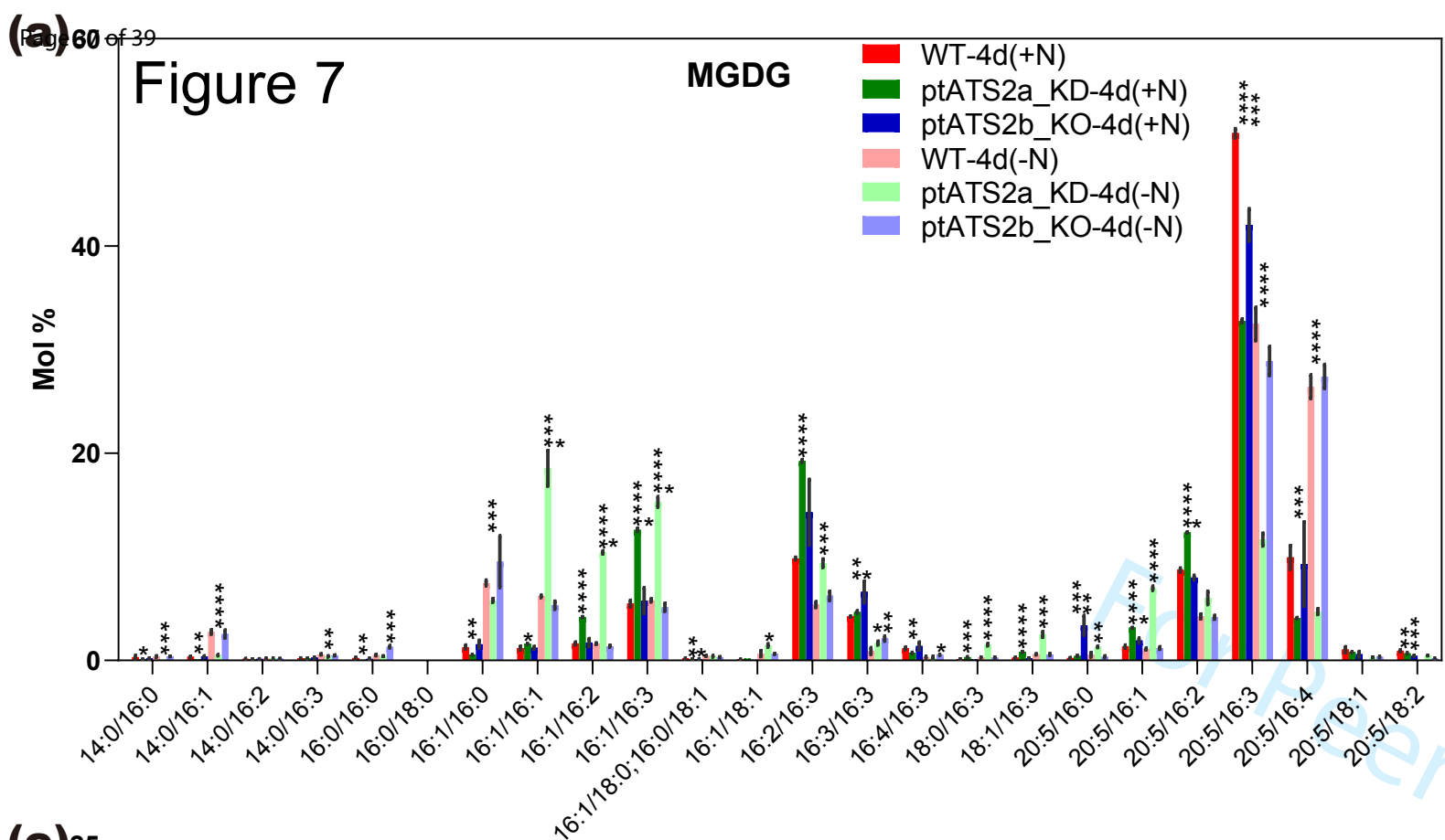
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WT	AAACTGGTATTCAATATCTCAAAGA	CGGGC	TTCCC	GAAAGGCACTCGCTCTCGTAC	CGGCAAGG -0 bp
7-2	AAACTGGTATTCAATATCTCAA	-----//	-----	-----	GGCAAGG -50 bp
7-6	AAACTGGTATTCAATATCTCAA	-----//	-----	-----	GGCAAGG -50 bp
11-2	AAACTGGTATTCAATATCTCAA	-----//	-----	-----	GGCAAGG -50 bp

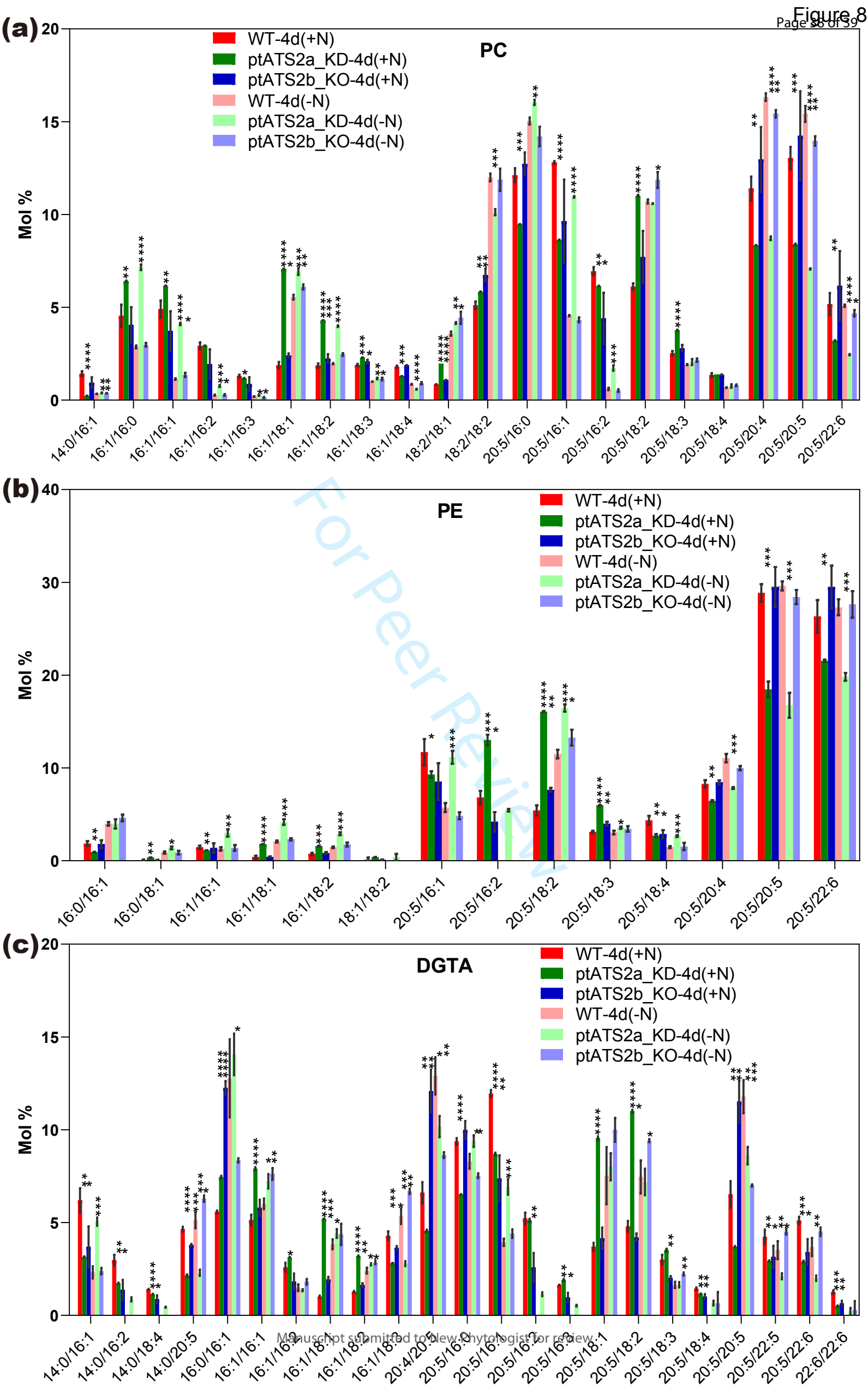
(f)

	20-bp gRNA target site	PAM	20-bp gRNA target site	PAM	
WT	TGCCATGTATTGGACAACAAC	TAGAGGGGGTATG	TCCGA	AAGGTATGCGCTCCCGCA	TGGAAAAC -0 bp
2-3	TGCCATGTATTGGACAA	-----//	-----	-----	ATGGAAAAC -250 bp
2-4	TGCCATGTATTGGACAA	-----//	-----	-----	ATGGAAAAC -250 bp
2-12	TGCCATGTATTGGACAA	-----//	-----	-----	ATGGAAAAC -250 bp









TAG

- WT-4d(+N)
- ptATS2a_KD-4d(+N)
- ptATS2b_KO-4d(+N)
- WT-4d(-N)
- ptATS2a_KD-4d(-N)
- ptATS2b_KO-4d(-N)

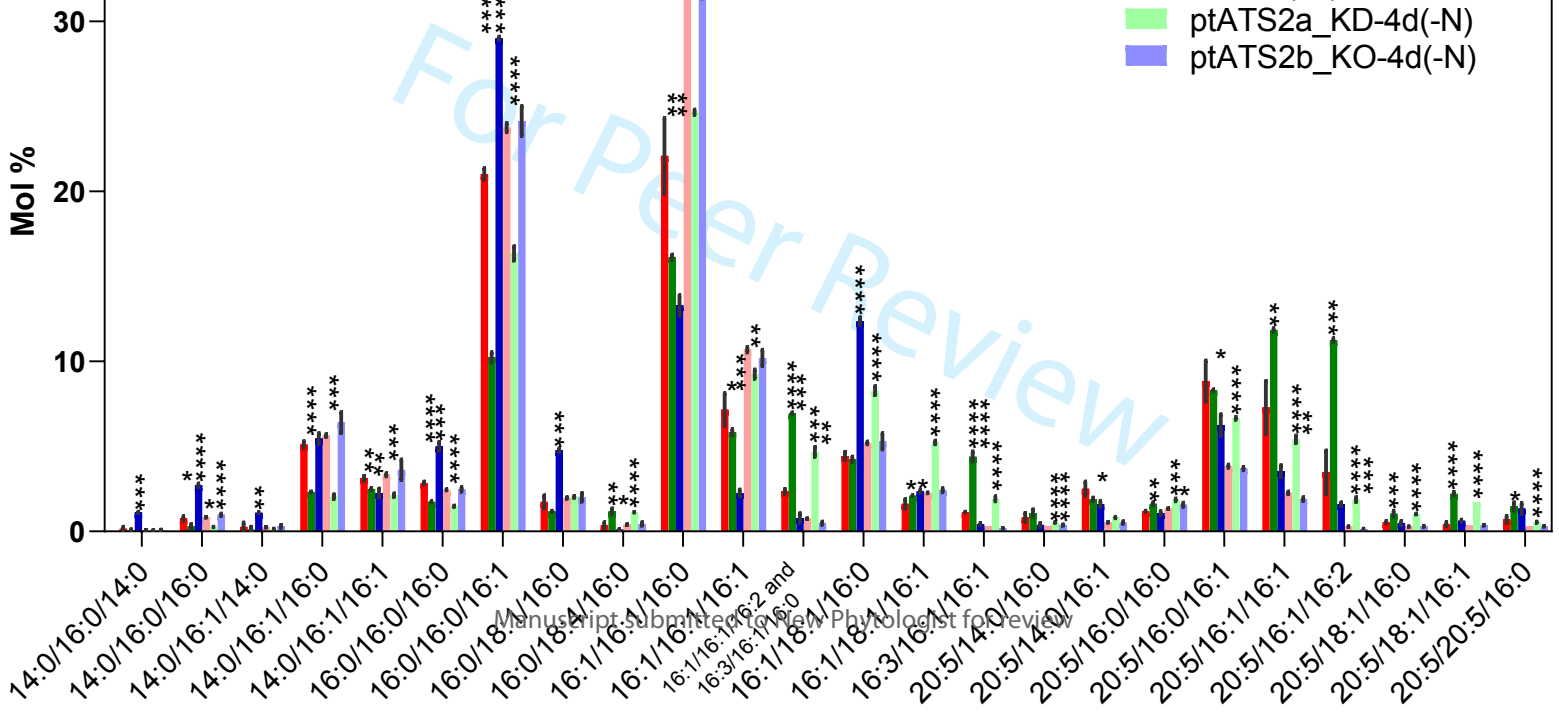


Figure 10

