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Plastidic $\Delta 6$ Fatty-Acid Desaturases with Distinctive Substrate Specificity Regulate the Pool of C18-PUFAs in the Ancestral Picoalga *Ostreococcus tauri*

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1 **PLASTIDIC $\Delta 6$ FATTY-ACID DESATURASES WITH DISTINCTIVE**
2 **SUBSTRATE SPECIFICITY REGULATE THE POOL OF C18-PUFAS IN THE**
3 **ANCESTRAL PICOALGA *OSTREOCOCCUS TAURI***

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15 ***Short title:*** *plastidic $\Delta 6$ -desaturase in the green lineage*

16 ***One sentence summary:*** *Osteococcus tauri plastidic lipid C18-PUFA remodelling*
17 *involves two plastid-located cytochrome-b5 fused $\Delta 6$ -desaturases with distinct preferences for*
18 *both head-group and acyl-chain.*

19 ***Footnotes:***

20 ***Author Contributions***

21 *CDG performed the work and analyses on O. tauri (cloning, transgenic screening, HP-*
22 *TCL, GC-FID, MS/MS); RD performed the work and analyses on N. benthamiana OE*
23 *(cloning, agro-transformation, FAMES analysis); CL performed most of the lipid analyses of*
24 *O. tauri and N. benthamiana (agro-transformation, HP-TLC, GC-FID); NP performed the*
25 *work and analysis on Synechocystis (transformation, screening, TCL, GC-FID); SM designed,*

26 performed and analyzed the photosynthesis experiments; KT performed cloning and qPCR
27 experiments; JeJ performed the work on DES localization and qPCR analyses; JuJ performed
28 MS/MS analyses; JG performed the work on DES localization; IS supervised the work on
29 *Synechocystis*; FD performed and supervised the work on *N. benthamiana*, helped to organize
30 the MS; FC designed, supervised and performed the research, analyzed the data (*O. tauri*, *N.*
31 *benthamiana*, *Synechocystis*), wrote the paper.

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33 this article in accordance with the policy described in the Instructions for Authors are:
34 Florence Corellou, florence.corellou@u-bordeaux.fr and Iwane Suzuki,
35 iwanes6803@biol.tsukuba.ac.jp concerning *Synechocystis* PCC 6803 lines.

36 ABSTRACT

37 Eukaryotic $\Delta 6$ -desaturases are microsomal enzymes which balance the synthesis of ω -3
38 and ω -6 C18-polyunsaturated-fatty-acids (PUFA) accordingly to their specificity. In several
39 microalgae, including *O. tauri*, plastidic C18-PUFA are specifically regulated by
40 environmental cues suggesting an autonomous control of $\Delta 6$ -desaturation of plastidic PUFA.
41 Sequence retrieval from *O. tauri* desaturases, highlighted two putative $\Delta 6/\Delta 8$ -desaturases
42 sequences clustering, with other microalgal homologs, apart from other characterized Δ -6
43 desaturases. Their overexpression in heterologous hosts, including *N. benthamiana* and
44 *Synechocystis*, unveiled their $\Delta 6$ -desaturation activity and plastid localization. *O. tauri* lines
45 overexpressing these $\Delta 6$ -desaturases no longer adjusted their plastidic C18-PUFA amount
46 under phosphate starvation but didn't show any obvious physiological alterations. Detailed
47 lipid analyses from the various overexpressing hosts, unravelled that the substrate features
48 involved in the $\Delta 6$ -desaturase specificity importantly involved the lipid head-group and likely
49 the non-substrate acyl-chain, in addition to the overall preference for the ω -class of the
50 substrate acyl-chain. The most active desaturase displayed a broad range substrate specificity
51 for plastidic lipids and a preference for ω -3 substrates, while the other was selective for ω -6
52 substrates, phosphatidylglycerol and 16:4-galactolipid species specific to the native host. The
53 distribution of plastidial $\Delta 6$ -desaturase products in eukaryotic hosts suggested the occurrence
54 of C18-PUFA export from the plastid.

55

57 Marine microalgae synthesize peculiar polyunsaturated fatty-acids including
58 hexatetraenoic acid, (16:4^{Δ4,7,10,13} HTA), stearidonic acid (SDA, 18:4^{Δ6,9,12,15}), octapentaenoic
59 acid (OPA18:5^{Δ3,6,9,12,15}) as well as very-long-chain polyunsaturated-fatty-acids (VLC-PUFA)
60 (Khozin-Goldberg *et al.*, 2016; Jonasdottir, 2019). Due to overfishing and pollution,
61 microalgae are regarded as a sustainable alternative for the production of health-beneficial
62 PUFA inefficiently produced by vertebrates, such as SDA and docosahexaenoic acid (DHA,
63 22:6^{Δ4,6,9,12,15}). However, still little is known about the molecular regulation of PUFA
64 synthesis in microalgae. SDA and γ -linolenic acid (GLA, 18:3^{Δ6,9,12}) are the Δ 6-desaturation
65 products of α -linolenic acid (ALA, 18^{Δ9,12,15}), and of linoleic acid (LA, 18:2^{Δ9,12}),
66 respectively. The substrate preference of Δ 6-desaturase (DES) is considered as the main
67 switch to direct C18-PUFA flows towards the ω -3 or the ω -6 pathways (Shi *et al.*, 2015). The
68 ω -3-desaturation of ω -6 substrates establishes a further link between the ω -6 and ω -3 pathway
69 in lower eukaryotes (Wang *et al.*, 2013). FA fluxes between lipids and compartments,
70 including cytosolic lipids droplets, are also known to participate in PUFA-remodeling of
71 structural lipid, though knowledge about these fluxes in microalgae remains scarce (Li-
72 Beisson *et al.*, 2015; Li, N *et al.*, 2016).

73 Nutrients and abiotic stresses regulate the PUFA content of cyanobacteria and microalgae
74 (Los *et al.*, 2013; Khozin-Goldberg *et al.*, 2016; Kugler *et al.*, 2019). In particular, C18-PUFA
75 from plastidic lipids are highly remodeled in response to abiotic stresses; in the cyanobacteria
76 *Synechocystis* sp. PCC6803, ALA and SDA synthesis is triggered by chilling while in several
77 species from the Chromista kingdom OPA is either increased or redistributed within
78 molecular species (Tasaka *et al.*, 1996; Kotajima *et al.*, 2014; Leblond *et al.*, 2019). As plants,
79 green microalgae display a high amount of α -linolenic acid (ALA, 18:3^{Δ9,12,15}) and further the
80 peculiar FA, 16:4^{Δ4,7,10,13} which is typical of the Chlorophyta phylum (Lang *et al.*, 2011).
81 Major microalga classes from the Chromista kingdom, such as haptophytes and
82 dinoflagellates, produce both OPA and DHA which are not synthesized by green microalgae.
83 SDA is usually predominant in those species and required for both the synthesis of OPA
84 found in galactolipids and of ω -3 VLC-PUFA, including DHA (Jonasdottir, 2019; Peltomaa *et*
85 *al.*, 2019).

86 *Ostreococcus tauri* is an ancestral green picoalga that emerged early after the divergence
87 between Chlorophyta and Streptophyta (land plants) (Courties *et al.*, 1994; Chrétiennot-Dinet
88 *et al.*, 1995; Leliaert *et al.*, 2012). It has the most minimal genomic and cellular organization
89 (Derelle *et al.*, 2006). This coccoid cell is smaller than 2 μm (picoeukaryote), lacks cell-wall,
90 flagella, as well as an obvious sexual life (Grimsley *et al.*, 2010). However, it displays a large
91 panel of PUFA, including HTA, ALA, SDA, OPA and DHA as main components (Wagner *et al.*
92 *et al.*, 2010). *O. tauri* glycerolipid characterization unveiled a clear-cut allocation of PUFA in
93 membranes, with C18-PUFA prevailing in plastidic lipids, OPA being restricted to
94 galactolipids, and VLC-PUFA exclusively found in the extraplastidic lipids consisting of the
95 betain lipid diacylglyceryl-hydroxymethyl-trimethyl- β -alanine (DGTA) and the
96 phosphosulfolipid phosphatidyl-dimethylpropanethiol (PDPT) (Degraeve-Guilbault *et al.*,
97 2017). We further showed that nutrient starvation resulted in the increase of ALA at the
98 expense of SDA in plastidic glycerolipids and reverberated in the acyl-CoA pool and in
99 triacylglycerols (TAG) with no significant impact in extraplastidic glycerolipids. The sole $\Delta 6$ -
100 DES previously characterized from *O. tauri* (and related species from the class
101 *Mamiellophyceae*) uses acyl-CoA instead of acyl-lipid as substrates, in contrast to other $\Delta 6$ -
102 DES from lower eukaryotes (Domergue *et al.*, 2005). We reasoned that the regulation of the
103 plastidic C18-PUFA pool involves uncharacterized acyl-lipid $\Delta 6$ -DES, rather than a transfer
104 of $\Delta 6$ -desaturation products from the acyl-coA pool to the chloroplast.

105 In this work, we identified two novel acyl-lipid $\Delta 6$ -DES, which were plastid located,
106 accepted plastidic lipids as substrates, and displayed distinctive specificities depending on
107 overlapping substrate features. Together with putative homologs from the Chromista and
108 Kinetoplastida lineages, these $\Delta 6$ -DES cluster apart from the previously characterized $\Delta 6$ -
109 DES. The discovery of plastidic $\Delta 6$ -DES and the impact of their overexpression in *O. tauri*
110 points out the requirement of tight regulation of the C18-PUFA pool in microalgae. (Lee *et al.*
111 *et al.*, 2016; Li, D *et al.*, 2016). The strategy used in this study further illustrates how
112 overexpression in several host systems of distinctive glycerolipid composition gives insight
113 into DES substrate specificity.

Thirteen canonical DES sequences were retrieved from genomic and transcriptomic databases (NCBI databases). All sequences were manually checked upstream of the predicted start codon, especially in order to assess the N-terminal (Nt) part of the proteins, and extended ORF were validated by cDNA amplification (Table S1, Table S2). Exception made of the acyl-CoA- Δ 6-DES and an unknown DES barely related to sphingolipid Δ 3/ Δ 4-DES, all DES were predicted to contain a chloroplastic target-peptide (cTP) (Tardif *et al.*, 2012). Among the seven front-end DES three uncharacterized Δ 6/ Δ 8 fatty-acid-DES retained our attention (Table S1, Fig. 1) (Kotajima *et al.*, 2014). One Δ 6-DES candidate clustered with acyl-lipid Δ 6/ Δ 8-DES and was closest to the diatom *Thalassiosira pseudonana* Δ 8-sphingolipid-DES (Tonon *et al.*, 2005). The two other candidates were closely related (49.8% identity, 71.6% similarity) and, together with putative homologs, formed a cluster apart from the typical acyl-CoA Δ 6-DES from *Mamiellophyceae* species and acyl-lipid Δ 6/ Δ 8-DES from plants, fungi and worms (Fig. 1A, Fig. S1 to S4). These two candidates had each one homolog in the *Mamiellophyceae* species (exception made of *Bathycoccus prasinus*) (Fig. S2, Fig. S3). More distantly related homologs occurred in microalgae arising from secondary endosymbiosis, i.e., species from the Chromista and Euglenozoa supergroup and not in the green lineage (Fig. S4). All homologous sequences displayed the typical Nt-fused-Cyt-b5 domain found in front-end DES. Three His-Boxes, which are known to be involved in both DES activity and specificity, were conserved (Fig. 1B, Fig. S2 to Fig. S4) (Sayanova *et al.*, 1997; López Alonso *et al.*, 2003). Consensus motifs emerging from alignments corresponded to xHDYxHGRx, WWSxKHNxHH, and QLEHHFLP with a larger conserved region upstream of this latter motif, and showed clearly divergent amino acid signature compared to the acyl-CoA- Δ 6-DES His-Boxes (QHEGGHSSL, WNQMHNKHH, QVIHHLFP) (Fig.1B, Fig. S2 to S4) (López Alonso *et al.*, 2003). *O. tauri* acyl-CoA- Δ 6-DES was previously characterized in yeast and extensively used for VLC-PUFA reconstruction pathway in various organisms including plants (Domergue *et al.*, 2005; Hoffmann *et al.*, 2008; Ruiz-López *et al.*, 2012; Hamilton *et al.*, 2016). However, its activity has never been assessed in the native host; The acyl-CoA - Δ 6-DES was therefore chosen as a reference to achieve the functional characterization of the two closely related Δ 6/ Δ 8-DES candidates. According to their genomic accessions, these

candidates will be referred to as Ot05 and Ot10 and the acyl-CoA- Δ 6-DES to as Ot13 (Table S1).

116 Δ 6-DES-candidate localization and activities in heterologous hosts

117 Full-length ORF or codon-optimized and ORF Nt-truncated, for removing the putative
118 cTP, were expressed in *S. cerevisiae*. Neither the supply of Δ 6-substrates nor of Δ 8-substrates
119 resulted in the detection of any products. The transformation efficiency was assessed by co-
120 transformation of candidates with the acyl-CoA Δ 6-DES, which results in the synthesis of Δ 6-
121 products from supplied Δ 6-substrates (Fig. S5). We therefore overexpressed full-length
122 proteins in *N. benthamiana* to assess their sub-cellular localization and test their putative Δ 6-
123 desaturation activity on the endogenous substrates 18:3n-3 and 18:2n-6. The two putative
124 p Δ 6-DES Ot05 and Ot10 fused to the -yellow fluorescent protein (Ct-YFP) exclusively
125 localized at plastids, while the acyl-CoA- Δ 6-DES-YFP was at the ER (Fig. 2A). Expression
126 of either fused and non-fused Δ 6-DES candidates and acyl-CoA- Δ 6-DES resulted in the
127 synthesis of the Δ 6-desaturation products 18:3n-6 and 18:4n-3 (Fig. 2B, Fig. S6A). These
128 results unambiguously demonstrated that both Ot05 and Ot10 are plastidic Δ 6-DES. A clear
129 trend emerged from the variation of the ω -3 and ω -6 C18-PUFA ratio in individual replicates
130 (Fig. 2B, Fig. S6B).; The 18:4n-3/18:3n-3 ratio was higher in Ot05 overexpressors (Ot05-OE)
131 while the 18:3n-6/18:2n-6 ratio was increased in Ot10-OE and especially Ot13-OE. This
132 suggests that Ot05 preferentially accepted ω -3 substrates while the specificity of Ot10 was
133 higher for ω -6 substrates. The ω -3 and ω -6 C18-PUFA ratio was also used to readily compare
134 the relative impact on glycerolipid classes of each Δ 6-DES-OE independently of the overall
135 activity (Fig. 2C, Fig. S7). As a result, extraplastidic phospholipids (phosphatidylcholine PC,
136 Phosphatidic Acid, PA, and phosphatidylethanolamine PE) were mostly impacted in the Ot13-
137 OE (acyl-CoA- Δ 6-DES) and the ω -6 ratio 18:3n-6/18:2n-6 was also importantly increased in
138 MGDG. The extraplastidic lipids were affected to a lesser extent in the p Δ 6-DES-OE Ot05
139 and Ot10 (Fig. 2C). Most interestingly, monogalactosyl diacylglycerol (MGDG) was the most
140 altered lipid class in Ot05-OE, while in Ot10-OE, PG showed the highest increase of Δ 6-
141 products/ Δ 6-substrates ratio in Ot10-OE. Impact on sulfoquinovosyl diacylglycerol (SQDG)
142 and digalactosyl diacylglycerol (DGDG) was greater in Ot05-OE compared to the two other
143 OE. Interestingly, disrupting the heme-binding capacity of Cyt-b5 by H>A mutation in the

144 HPGG motif, abolished the activity of both plastidic $\Delta 6$ -DES (p $\Delta 6$ -DES) (Sayanova *et al.*,
145 1999) (Fig. S6C).

146 To further clarify the substrate specificity of p $\Delta 6$ -DES, the cyanobacterium *Synechocystis*
147 PCC 6803 was used. This organism not only encompasses the eukaryotic classes of plastidic
148 lipids as major glycerolipids but also allows transgene expression from a similar genomic
149 environment (homologous recombination). *Synechocystis* PCC 6803 has a $\Delta 6$ -DES (*desD*)
150 and a ω -3-DES (*desB*). In wild type (WT), the selectivity of DesD for galactolipids is
151 reflected by the exclusive distribution of 18:3n-6 in galactolipids. The transcription of *desB* is
152 induced at temperatures below 30°C and results in 18:3n-3 accumulation in PG and SQDG,
153 and of 18:4n-3 in galactolipids. Note that all glycerolipid species are *sn-1/sn-2* 18:X/16:0
154 combinations.

155 Complementing either Ot05 or Ot10 in Δ *desD* cells of *Synechocystis* at 34°C (no endogenous
156 ω -3-DES activity) resulted in 18:3n-6 production at the expense of 18:2n-6 (Fig. 3A). Similar
157 to experiment in *N. benthamiana*, overexpression of H/A mutated version of the $\Delta 6$ -DES
158 resulted in the absence of $\Delta 6$ product, indicating that the integrity of the HPGG motif in the
159 Cyt-b5 domain is required. Ot05 overexpression restored the WT FA-profile, while Ot10
160 overexpression relatively weak effect. Most interestingly, accumulation of 18:3n-6 occurred
161 not only in galactolipids but was also detected in SQDG for Ot05-OE and in PG for both
162 Ot05-OE and Ot10-OE (Fig. 3B, Fig. 3C, Fig. 3D). Noteworthy, 18:3n-6 accumulation in PG
163 for Ot10-OE was twice as high as that for Ot05-OE, and an isomer of 18:2, likely
164 corresponding to the $\Delta 6$ desaturation product of 18:1n-9, was further specifically produced
165 (Fig. 3D). Since no acyl-editing unlikely occurs in cyanobacteria, these evidences support that
166 in addition to galactolipids, SQDG and PG are substrates of Ot05, and PG is a preferential
167 substrate of Ot10.

168 *DesB* induction where *Synechocystis* was grown at 24°C led to 18:3n-3 synthesis and
169 further 18:4n-3 accumulation in galactolipids. The increase of 18:3n-6 at the expense of
170 18:2n-6 reflects that the endogenous $\Delta 6$ -desaturation of 18:2n-6 is favored and that $\omega 3$ -
171 desaturation is a limiting step for 18:4n-3 production in WT (Fig. 3E). Overexpression of *O.*
172 *tauri* p $\Delta 6$ -DES restored the production of 18:4n-3 in the Δ *desD* background to a similar
173 degree in Ot10-OE as in WT, and to a greater extent in Ot05-OE (Fig. 3E). By comparing the
174 profiles of Ot05-OE with WT, it appeared that 18:3n-6 was decreased to a greater extend and

175 that 18:3n-3 was depleted, indicating that the 18:2n-6, 18:3n-3, 18:4n-3 route prevailed over
176 the 18:2n-6, 18:3n-6, 18:4n-3 route. This is coherent with the results obtained in *N.*
177 *benthamiana*. Interestingly, 18:4n-3 not only accumulated in galactolipids of both pΔ6-DES
178 OE, but was also detected in PG-species and specifically in SQDG for Ot05-OE (3% of
179 SQDG species) (Fig. 3F, Fig. 3G, Fig. 3H). While 18:X/16:0 galactolipids and SQDG species
180 appeared to be converted more efficiently by Ot05, 18:3n-3-PG seems to be equally well
181 accepted by both pΔ6-DES (Fig. 3D, Fig. 3H).

182 Δ6-DES overexpression in *Ostreococcus tauri*

183 To gain insight into the regulation of C18-PUFA pool by Δ6-DES in the native host, *O.*
184 *tauri* overexpressors of each Δ6-DES were created using the pOtOXLuc vector (Moullager *et*
185 *al.*, 2010).

186 Screening and selection of Δ6-DES transgenic lines

187 Phosphate limitation is required for the maximal activity of the high-affinity-phosphate-
188 transporter promoter (promHAPT) driving transgene overexpression. Furthermore, phosphate
189 limitation has been previously shown to enhance the accumulation of 18:3n-3 at the expense
190 of 18:4n-3 in WT (Degraeve-Guilbault *et al.*, 2017). Transgenics for each of the Δ6-DES
191 were screened by luminescence recording of the luciferase reporter gene (promCCA1:Luc)
192 and their FA-profiles were further assessed (Fig. S8). Five transgenics with various
193 phenotypes were selected to ascertain the FA-profile and the transgene expression level (Fig.
194 S9). The transgenics displaying the highest luminescence levels showed the most pronounced
195 alterations regarding the amount of C18-PUFA (Fig. 4). In Ot05 transgenics lines 5-3, 5-4 and
196 5-5, 18:4n-3 was greatly increased at the expense of 18:3n-3, and a less drastic increase of the
197 down-product 18:5n-3 was observed. These variations on ω-3-C18-PUFA were less
198 pronounced in the best Ot10 transgenics (10-2, 10-5), while the impact of Ot05 and Ot10
199 overexpression on ω-6-C18-PUFA was comparable (Fig. 4A, 4B and 4C, Fig. S9B). Only
200 minor alterations, consisting mainly of 18:3n-3 reduction, were observed for Ot13-OE (Fig. 4,
201 Fig. S9). Additional changes, such as a decrease of 16:4n-3 and a slight increase of 20:4n-6,
202 were observed in the best pΔ6-DES-OE (Fig. S9, Fig. 4D). One representative overexpressor
203 for each Δ6-DES was chosen (Ot05-5, Ot10-5, and Ot13-5, which displayed a similar level of

204 transgene expression) were further used for detailed analysis (Fig. 4D, Fig. S10A). These
205 overexpressors grew similarly as control lines (Fig S10B).

206 *Lipidic features of selected $\Delta 6$ -DES overexpressors*

207 For $\Delta 6$ -DES OE, minor changes were observed in the proportion of lipid classes consisting
208 mainly of an increased accumulation of TAG, mostly at the expense of MGDG (Fig. S10C).
209 As expected, plastidic lipid FA-profiles were greatly impacted by p $\Delta 6$ -DES overexpression
210 while only slightly by the acyl-CoA- $\Delta 6$ -DES overexpression (Fig. 5). Overall FA variations
211 in plastidic lipids followed a similar trend in both p $\Delta 6$ -DES-OE: ω -6-C18-PUFA were
212 equally impacted in Ot05-OE and Ot10-OE, while the accumulation of 18:4n-3 or of its $\Delta 3$ -
213 desaturation product 18:5n-3 in 18:5/16:4-MGDG was greater for Ot05-OE (Fig. 5A, B).
214 Ot05-OE also displayed a specific and important decrease of the relative amount of 16:4n-3 in
215 DGDG that was paralleled by an increase of 16:0; however the proportion of 16:4n-3
216 remained stable in MGDG (Fig. 5A, 5C). Most interestingly molecular species analysis
217 unveiled that the differences between Ot05-OE and Ot10-OE were enhanced for the DGDG
218 species 32:4, 34:4 (18:X/14:0, 18:X-16:0) and the SQDG species 34:4. The relative
219 accumulation of 18:4/saturated fatty-acid combinations in Ot05-OE (ratio to control line) was
220 more than twice as high in DGDG and three times higher in SQDG as for Ot10-OE (Fig. 5D,
221 5F). In contrast, the variations of C18-PUFA from unsaturated galactolipids species were
222 close to one another in Ot05-OE and Ot-10-OE.

223 Extraplasmidic structural lipids showed modest alterations consisting mainly of a 30%
224 increase of the proportion of 20:4n-6 in the three $\Delta 6$ -DES OE (Fig. 5G). Under phosphate
225 limitation, DGTA is the prevailing extraplasmidic structural lipid (Degraeve-Guilbault *et al.*,
226 2017) (Fig. S11A, S11B). A specific increase of 18:4n-3 was observed in DGTA for Ot05-OE
227 and was likely relying on the increase of the species 32:4 which includes 14:0/18:4-DGTA
228 (Fig. 5G, Fig. S11C). Similar to the phenotype observed in DGDG, 16:4n-3 was more
229 importantly decreased in Ot05-OE. Several molecular species specifically decreased in Ot05-
230 OE, including 36:8 (putatively 20:4/16:4), 30:4 (14:0/16:4), and 38:10 (22:6/16:4), might
231 account for the overall 16:4 decrease.

232 Overexpression of each of the three $\Delta 6$ -DES-OE importantly impacted the FA-profile and
233 molecular species of TAG (Fig. 5H, Fig. S12, S13). Notably for Ot13-OE, the clear alteration

234 of the ratio $\Delta 6$ -DES-substrates / $\Delta 6$ -DES-products in the TAG contrasted with the minor
235 alterations detected in structural lipids. As expected, all 18:4-containing species increased at
236 the expense 18:2- and/or 18:3-TAG species in $\Delta 6$ -DES-OE: for instance, the major molecular
237 TAG species 48:4, 50:4 increased while the major 48:3, 50:3 species decreased (Fig. S12,
238 S13). Noteworthy, the peculiar species 50:10 (16:4/16:3/18:3 and possibly 16:4/16:4/18:2)
239 and 50:11, (16:4/16:4/18:3) were the second most importantly reduced species. As previously
240 described for DGTA and DGDG, the relative amount of 16:4n-3 was specifically decreased in
241 Ot05-OE, while 16:0 was increased (Fig. 5H). Other alterations specific to Ot05-OE consisted
242 of the reduction of the species 48:7 (includes 16:4/14:0/18:3), 50:6 (includes 16:4/16:0/18:2)
243 and 50:7 (includes 16:4/16:0/18:3), by about half, and the increase of the proportion of 54:10
244 (includes 18:4/14:0/22:6), 56:9 (includes 18:4/16:0/22:5) and 56:10 (18:4/16:0/22:6) by more
245 than twice. Therefore, the specific 16:0 increase in TAG from Ot05-OE most likely relies on
246 56:9 and 56:10; these species are 16:0 *sn*-2-TAG species, i.e., TAG species possibly arising
247 from plastidic DAG precursors (Degraeve-Guilbault *et al.*, 2017). Altogether, these
248 observations indicate that FA fluxes toward TAG are differentially affected in the $\Delta 6$ -DES-
249 OE, and further suggest that 16:4-TAG species, including the peculiar di-16:4 species, are
250 importantly involved in the fine-tuning of C18-PUFA.

251 *Physiological relevance of p $\Delta 6$ -DES regulation*

252 Transcriptional regulation of desaturases is known to occur in response to environmental
253 cues. We therefore assessed transcript levels of desaturases involved in the regulation of C18-
254 PUFA pool by phosphate availability, including the putative $\omega 3$ -DES (Kotajima *et al.*, 2014).
255 Consistent with our previous report, the proportion of 18:3n-3 was increased by about one
256 half after the transfer of cells to phosphate depleted medium (Fig. 6A). By that time, the
257 transcript level of Ot05 was decreased by more than 60%, the transcripts levels of Ot10 and of
258 the putative ω -3-DES remained stable while transcripts of the acyl-CoA DES were rather
259 increased. This result indicates that a decrease in Ot05 activity through transcriptional
260 repression results in lowering the 18:4n-3/18:3n-3 ratio under phosphate deprivation.

261 Thylakoid membrane PUFA are known to play a role in the regulation of photosynthetic
262 processes (Allakhverdiev *et al.*, 2009). We therefore investigated photosynthetic parameters
263 of $\Delta 6$ -DES-OE. Nevertheless, no significant changes regarding either photosynthesis
264 efficiency or photoinhibition responses occurred under our conditions (Fig. 6C, S14)

265 DISCUSSION

266 Regulation of C18-PUFA desaturation is required for the FA-profile remodelling of
267 structural lipids in response to chilling in plants and cyanobacteria (Los *et al.*, 2013);
268 downstream synthesis of ω -3 and ω -6 VLC-PUFA in animals, fungi and microalgae also
269 involves the fine-tuning of C18-PUFA amount notably by Δ 6-DES. On the other hand, all
270 front-end Δ 6-DES studied so far are demonstrated, or assumed to be located in the ER
271 (Meesapyodsuk & Qiu, 2012). By unveiling the occurrence of plastidic Δ 6-DES with distinct
272 substrate selectivity in the ancestral green picoalga *O. tauri* and of putative homologs in
273 Chromista, our results strongly suggest the requirement of an autonomous control of plastidic
274 C18-PUFA in several microalgae species. The entangled substrate features instructing the
275 activity of these two novel Δ 6-DES, the possible PUFA fluxes unveiled by Δ 6-DES
276 overexpression, as well as the physiological significance of p Δ 6-DES are discussed.

277 Substrate specificity of *O. tauri* Δ 6-DES

278 DES specificity relies on intricate substrate features, including the acyl-chain position,
279 length, unsaturation features, and the acyl-carrier nature (Heilmann *et al.*, 2004a; Li, D *et al.*,
280 2016). Domain swapping between DES of distinctive specificity, together with further amino
281 acid mutation, could highlight the primary importance of His-Box and surrounding region for
282 front-end desaturase activity and substrate specificity (Song *et al.*, 2014; Li, D *et al.*, 2016;
283 Watanabe *et al.*, 2016). However, neither the exact molecular features underlying DES
284 (regio)specificity nor the hierarchical importance of the substrate features are yet clearly
285 identified. Furthermore, assaying plant DES activity in *S. cerevisiae* might have introduced
286 some bias by favoring the activity of microsomal desaturases and/or hampering the proper
287 analysis of plastidic desaturases specificity in the absence of plastidic substrate (Heilmann *et*
288 *al.*, 2004b).

289 In this work, four different hosts were used to characterize p Δ 6-DES substrate specificity.
290 Lipid changes occurring in each of these organisms reflect a steady-state arising from both
291 desaturation and overall FA fluxes. Nevertheless, comparison of lipidic features triggered by
292 overexpression of each Δ 6-DES in a given host and of the same Δ 6-DES in different hosts
293 allowed to gain insight into p Δ 6-DES substrate specificity. In 16:3-plants, the Kennedy
294 pathway contributes to the synthesis of plastidic lipid synthesis yielding 18:3/18:3-lipid

295 species besides *sn-1/sn-2* 18:3/16:3n-3 (Browse *et al.*, 1986b). In *O. tauri*, as in cyanobacteria
296 and other most green microalgae, plastidic lipids correspond to *sn-1/sn-2* 18:X/16:X species.
297 In contrast, *O. tauri* extraplastidic lipids encompass *sn-1/sn-2* SFA/16:4, VLC-PUFA/16:4
298 and di-homo-VLC-PUFA as major species (Degraeve-Guilbault *et al.*, 2017). These
299 distinctive positional signatures strongly suggest that, in *O. tauri*, plastidic lipids synthesis is
300 independent of ER synthesis. On the other hand, acyl-lipid remodelling of plastidic lipids is
301 assumed to be absent in *Synechocystis* PCC 6803, for which no acyl-turnover was ever
302 reported (Ohlrogge & Browse, 1995). Concerning microalgae, MGDG has been proposed, yet
303 not clearly proven, to be a platform of FA exchange supporting the incorporation of plastidic
304 FA into TAG in *C. reinhardtii* (Li *et al.*, 2012; Kim *et al.*, 2018). The interpretation of our
305 results takes support of this knowledge.

306 *Head-group specificity*

307 As major changes occurred in galactolipids independently of the host, it can reasonably be
308 concluded that at least MGDG is a substrate of both Ot05 and Ot10. Most interestingly,
309 compared to Ot10 overexpression, Ot05 overexpression in *O. tauri*, *Synechocystis* PCC 6803
310 and *N. benthamiana* triggered a greater/exclusive accumulation of $\Delta 6$ -DES products in SQDG
311 whereas Ot10 overexpression in *Synechocystis* PCC 6803 and *N. benthamiana*, led to a
312 greater accumulation of 18:3n-6 in PG. Ot10 overexpression further led to the production of
313 18:2-PG in *Synechocystis*. Altogether, these results show that Ot05 displayed a broad
314 specificity for plastidic substrates while Ot10 appeared to be selective for PG, at least in
315 heterologous hosts, as well as of some galactolipid species in the native host (see below).
316 Interestingly, the plastidic ω -3-DES FAD8 was shown to display a preference for PG
317 compared to the closely related FAD7 in *A. thaliana* (Roman *et al.*, 2015).

318 Concerning the impact of p $\Delta 6$ -DES overexpression on structural extraplastidic lipids,
319 several evidences support that it most likely arises from the export of overproduced PUFA
320 rather than from the access of p $\Delta 6$ -DES to extraplastidic substrates. Indeed, the absence of
321 desaturation activity in yeast supports that PC is not an accurate substrate (Domergue *et al.*,
322 2003). Moreover, the fact that *Ostreococcus* $\Delta 5$ -DES and $\Delta 4$ -DES, which natural substrates
323 are DGTA and possibly PDPT, displayed a low activity in *S. cerevisiae* supports the
324 importance of the head-group for front-end DES activity (Hoffmann *et al.*, 2008; Ahmann *et*
325 *al.*, 2011). We therefore propose that the first level of substrate recognition of *O. tauri* p $\Delta 6$ -

326 DES relies on plastidic lipid head-group. Recently, co-crystallization of the Stearoyl-CoA
327 DES with its substrate revealed that interaction between the DES and the acyl-carrier was
328 indeed fundamental to orient the acyl-chain in the catalytic tunnel of the enzyme (Wang *et al.*,
329 2015).

330 Acyl-CoA- $\Delta 6$ -DES overexpression in *O. tauri* importantly altered FA-profile of TAG and
331 that of structural lipids only moderately. In contrast, acyl-CoA- $\Delta 6$ -DES overexpression in *N.*
332 *benthamiana* resulted in the accumulation of $\Delta 6$ -desaturation products in all
333 phospholipids. These results are coherent with the acyl-CoA specificity of Ot13: in *O. tauri*
334 the incorporation of unwanted acyl-CoA $\Delta 6$ -DES products into TAG possibly circumvents
335 the alteration of extraplastidic lipids whereas in *N. benthamiana* leaves acyl-CoA are likely
336 preferentially incorporated into PC, and in part transferred to MGDG (see below).

337 *ω -3/ ω -6 and 16:4-galactolipid species selectivity*

338 The preference of Ot05 for ω -3-substrates was reflected by the important accumulation of
339 18:4n-3 at the expense of 18:3n-3 observed in Ot05-OE from all of the host organisms.
340 Conversely, Ot10 appeared globally more selective for ω -6 substrates. Nevertheless, Ot10
341 seemed to further display a ω -3 specificity for 16:4-galactolipid in which a rise of 18:4n-3
342 was paralleled by a drop of 18:3n-3. The ω -3-desaturation of the overproduced 18:3n-6 in
343 Ot10-OE could possibly be involved in the rise of 18:4n-3. However, one would expect a
344 higher accumulation of 18:3n-6 in Ot10-OE compared to Ot05-OE if only this route was used,
345 which is not the case. Indeed, the endogenous ω -3-desaturase appeared to compete efficiently
346 with the $\Delta 6$ -desaturation of 18:2n-6 to 18:3n-6 maintaining a high amount of 18:3n-3 in p $\Delta 6$ -
347 DES-OE; therefore, the specific 18:4n-3 accumulation in 16:4-galactolipids most likely
348 involves the $\Delta 6$ -desaturation of 18:3n-3 precursors in the Ot10-OE *O. tauri* line.

349 In summary our results suggest that in addition to a distinctive preference of Ot05 and
350 Ot10 for ω -3 substrates, Ot10 is further able to accept highly unsaturated galactolipids ω -3
351 substrates. Compared to *Synechocystis*, the higher activity of Ot10 on ω -6-substrates in *N.*
352 *benthamiana* and *O. tauri* might also rely on the higher unsaturation degree of the non-
353 substrate acyl-chain in these hosts. Therefore, the overall unsaturation degree of substrate
354 molecular species appears to influence the activity/ ω -specificity of Ot10 while Ot05 activity
355 seems independent of the unsaturation degree of the associated C16.

356 Export of plastidic PUFA

357 Overexpression of each of the three $\Delta 6$ -DES in *O. tauri* led to a similar increase of 20:4n-6
358 in DGTA and TAG and a specific increase of 18:4n-3 in DGTA for Ot05-OE. We previously
359 reported that the acyl-CoA pool was enriched in 18:3n-3 and 18:4n-3, whose amounts were
360 varying according to the plastidic C18-PUFA content (Degraeve-Guilbault *et al.*, 2017).
361 Together with the discovery of p $\Delta 6$ -DES, these previous observations support that $\Delta 6$ -
362 desaturation products are exported from the plastid to the acyl-CoA pool. Though it cannot be
363 excluded that p $\Delta 6$ -DES have (limited) access to extraplastidic substrates as suggested for the
364 plastidic ω -3-DES *C. reinhardtii*, front-end DES specificity is known to be more restricted
365 than those of ω 3-DES (Meesapyodsuk & Qiu, 2012; Nguyen *et al.*, 2013; Wang *et al.*, 2013).
366 Unexpectedly, p $\Delta 6$ -DES-OE impacted extraplastidic lipids to a much greater extent in *N.*
367 *benthamiana*. As mentioned above, these changes are likely arising from reallocation of
368 xenobiotic plastidic $\Delta 6$ -desaturation products to other membranes, possibly to circumvent
369 deleterious effects, and from the low capacity of leaves to synthesize TAG. In *A. thaliana*
370 mutants deficient for the plastidic ω -3-DES (*FAD7*), alteration of 18:3 content in
371 extraplastidic lipids has been reported and implied a two-way exchange of lipids between the
372 chloroplast and the extrachloroplastic compartments (Browse *et al.*, 1986a). Most of the
373 following work focused on the transfer of lipid and PUFA from the ER to the chloroplast,
374 establishing the idea that the reverse transport was negligible (Miquel & Browse, 1992; Li, N
375 *et al.*, 2016). Nevertheless, the recent characterization of two *Arabidopsis* plastid lipases
376 mutants highlighted that plastidic PUFA indeed contributed to PUFA remodelling of
377 extraplastidic lipid (Wang *et al.*, 2017; Higashi *et al.*, 2018).

378 Co-regulation of DGDG and DGTA PUFA content

379 In *O. tauri* 16:4 is exclusively at *sn*-2 position in both extraplastidic and plastidic lipids
380 while being absent from the acyl-CoA pool and mostly at lateral positions in TAG. The
381 SFA/16:4 combinations account for more than 70% and 20% of PDPT and DGTA species,
382 respectively (Degraeve-Guilbault *et al.*, 2017). In Ot05-OE, the concomitant 18:4n-3 increase
383 and 16:4n-3 decrease specifically observed in DGDG and DGTA again rises the question
384 about the origin of 16:4 extraplastidic again; indeed, the decreases of 20:4/16:4 DGTA and
385 18:3/16:4 DGDG could be related assuming that 16:4-DGDG species would yield DAG
386 precursors for 16:4-extraplastidic species synthesis. Alternatively, the TAG pool could be

387 involved. For instance, the peculiar TAGs species 16:4/16:4/18:X possibly gives DAG
388 precursors with a 16:4-*sn*-2. Galactosyl-Galactosyl-Galactolipid-transferase, yet not identified
389 in microalgae, supports the production of DAG from DGDG in plants under freezing
390 conditions (Moellering *et al.*, 2010). Export of specific DGDG-species to extraplastidic
391 membranes has been reported for plants and microalgae under phosphate starvation (Jouhet *et*
392 *al.*, 2004; Khozin-Goldberg & Cohen, 2006).

393 Significance of plastidic Δ 6-DES

394 The absence of alteration in growth or photosynthetic processes in *O. tauri* Δ 6-DES-OE is
395 possibly related to compensatory mechanisms, including the increase of 16:0 and the decrease
396 of 16:4. Photosynthesis defects were not detected in *Synechocystis* mutant devoided of 18:3n-
397 3 and 18:4n-4, and could be unveiled only in very specific conditions in the *Arabidopsis*
398 mutant lacking trienoic PUFA (Gombos *et al.*, 1992; Vijayan & Browse, 2002). There is
399 overall only little evidence that plastidic PUFA support photosynthetic processes (Mironov *et*
400 *al.*, 2012; Kugler *et al.*, 2019).

401 The only plastidial front-end desaturase so far described was a Δ 4-DES from *C. reinhardtii*
402 and its Cyt-b5 domain was shown to be active *in vitro* (Zauner *et al.*, 2012). Our data further
403 show that a functional Cyt-b5 is absolutely required for *O. tauri* p Δ 6-DES activity in both *N.*
404 *benthamiana* and *Synechocystis*. These results illustrate the very tight co-evolution of Cyt-b5
405 and desaturase domains (Napier *et al.*, 2003). It further indicates that a redox partner different
406 from the eukaryotic Cyt-b5 oxidoreductase is involved (Napier *et al.*, 2003; Kumar *et al.*,
407 2012; Meesapyodsuk & Qiu, 2012). One possible candidate is the Ferredoxin NADP⁺-
408 reductase (Yang *et al.*, 2015).

409 The requirement of three Δ 6-DES in an photosynthetic organism displaying the most
410 reduced set of genes, points to the necessity of regulating the chloroplast and the cytosolic
411 C18-PUFA pools distinctively. The existence of putative homologs of p Δ 6-DES in other
412 microalgae species, such as haptophytes and dinoflagellates, might also be related to the co-
413 occurrence of 18:5 in galactolipids and the prevalence of VLC-PUFA in extraplastidic lipids.
414 For the diatom *P. tricornutum*, the putative p Δ 6-DES homolog might be involved in the
415 desaturation of plastidic C16-PUFA as it had been suspected long ago (Domergue *et al.*,
416 2002). For most *Mamiellophyceae*, with the exception of *Bathycoccus prasinos*, the two p Δ 6-

417 DES most likely arise from gene duplication. Ot10 would have evolved to restrict its
418 specificity to a particular set of substrates. Transcriptional regulation of Ot05 by phosphate
419 deprivation indicates that it is a physiological target under these conditions. The selectivity of
420 Ot05 for SQDG, which is considered as a surrogate of PG under phosphate limitation,
421 supports the importance of Ot05 in these conditions. Ot10 and the putative ω -3-DES
422 transcriptional regulation might be targeted by other environmental cues, which still need to
423 be discovered.

424 **METHODS**

425 All chemicals were purchased from Sigma Chemical (St. Louis, MO, USA), when not
426 stated otherwise.

427 Biological material & cultures

428 *O. tauri* (clonal isolate from OtH95) was grown as previously described in artificial sea-
429 water with either 5 μ M or 35 μ M NaH₂PO₄; penicillin (0,5 mg/ml) and streptomycin (0.25
430 mg/ml) together with centrifugation cycles (1000g, 5min) were used to reduce bacterial
431 contamination; flow cytometry was used to assess growth and bacterial contamination
432 (Degraeve-Guilbault *et al.*, 2017). *Synechocystis* PCC 6803 was grown accordingly to
433 (Kotajima *et al.*, 2014). *N. benthamiana* plants were cultivated in a greenhouse under
434 controlled conditions (16h:8h photoperiod, 25°C). *Agrobacterium tumefaciens* strain GV3101
435 was grown in Luria Broth medium at 30°C; *Saccharomyces cerevisiae* strain InvSc1 (*MATA*
436 *adc2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52*; Invitrogen) was grown in synthetic dextrose
437 medium (5 mL, 50-mL Erlenmeyer flask, 30 °C, 180 rpm).

438 Cloning strategy

439 PCR amplifications of DES ORF were achieved using Q5® Polymerase by two-step PCR
440 on cDNA matrix (primers Table S4). Monarch DNA Gel Extraction kit was used when
441 necessary (New England Biolabs, Ipswich, MA, US). Overexpression vectors were the
442 pOtoxLuc for *O. tauri* (Moulager *et al.*, 2010), pTHT2031S vector for *Synechocystis* PCC
443 6803 (Kotajima *et al.*, 2014), the GATEWAY destination vectors pVT102-U-GW for *S.*
444 *cerevisiae* (Domergue *et al.*, 2010) and PK7W2G2D for *N. benthamiana* (Karimi *et al.*, 2002).
445 For subcellular localization, the final destination vector was pK7YWG2 (Karimi *et al.*, 2002)

446 N-terminal-YFP-fusion was used. Subcloning was achieved in pGEMT vector by restriction
447 enzymes (Promega, Madison, WI, US), in pUC57 for codon-optimized sequence used in
448 *Synechocystis* and for *S. cerevisiae* (GenScript Biotech, Netherlands) and/or in pDONR 221
449 for GATEWAY cloning. Restriction cloning was used for cloning in pOtLux, and ligation
450 was used to introduce the synthetic gene in pTHT2031S In-Fusion® HD cloning kit (Takara
451 Bio, Kusatsu, Japan). Sequencing was achieved by Genwiz (Genwiz, Leipzig, Germany).

452 Site-directed mutagenesis H >A of the HPGG motif of the Cyt-b5 domain was either
453 performed by Genescript (*N. benthamiana*) or using In-Fusion® HD cloning kit (Takara Bio,
454 Kusatsu, Japan) for *Synechocystis* after amplification using two mutagenic complementary
455 primers for amplifying pTHT2031-*Ot5H46A-S* and pTHT2031-*Ot10H20A-S* from
456 pTHT2031-*Ot5-S* and pTHT2031-*Ot10-S*, respectively. The mutated DNA sequence was
457 validated for the correct modification using BigDye® Terminator v.3.1 (Life Technologies,
458 Carlsbad, CA, US).

459 RNA and cDNA preparation and quantitative RT-PCR analysis

460 RNeasy-Plus Mini kit (Qiagen, Hilden, Germany) was used for RNA purification; DNase I
461 was used to remove contaminating DNA (DNA-free kit, Invitrogen, Carlsbad, USA) and
462 cDNA obtained using the reverse transcription iScript™ supermix kit (Bio-Rad, Hercules,
463 CA, USA). Real-time RT quantitative PCR reactions were performed in a CFX96™ Real-
464 Time System (Bio-Rad) using the GoTaq® qPCR Master mix (Promega, Madison, WI, USA)
465 (Primers Table S4). Bio-Rad CFX Manager software was used for data acquisition and
466 analysis (version 3.1, Bio-Rad). Ct method was used to normalized transcript abundance with
467 the references mRNA *EF1α* (elongation factor), *CAL* (calmodulin), and *ACTprot2* (Actin
468 protein-related 2). PCR efficiency ranged from 95 to 105%. Technical triplicate was used, and
469 at least two independent experiments were achieved.

470 Genetic transformation

471 *O. tauri* electroporation was adapted from (Corellou *et al.*, 2009). Transgenics were
472 obtained by electroporation and pre-screened accordingly to their luminescent level
473 (Moulager *et al.*, 2010). *S. cerevisiae* was transformed using a PEG/lithium acetate protocol,
474 and FA supplementation was achieved as previously described (Dohmen *et al.*, 1991). Control

475 lines are transgenics of empty vectors. *N. benthamiana* leaves from five-week old plants were
476 infiltrated with *Agrobacterium tumefaciens* previously transformed by electroporation; the
477 p19 protein to minimize plant post-transcriptional gene silencing (PTGS) was used in all
478 experiments (Voinnet *et al.*, 2003). Briefly, *A. tumefaciens* transformants were selected with
479 antibiotics (gentamycin 25µg/mL with spectinomycin 100µg/mL or kanamycin 50µg/mL).
480 *Agrobacterium* transformants were grown overnight, diluted to a OD600 to 0.1 and grown to
481 a OD600 of 0.6-0.8. Cells were re-suspended in 5 mL sterilized H₂O for a final OD of 0.4 and
482 0.2 for overexpression and subcellular localization experiments, respectively and 1 mL was
483 agroinfiltrated. Plants were analyzed 2 and 5 days after *Agrobacterium* infiltration for
484 subcellular localization experiments and for overexpression, respectively.

485 *Synechocystis* transformation was achieved by homologous recombination (Williams,
486 1988). Briefly, the plasmid was transformed into ten-time concentrated cells of the $\Delta desD$
487 strain collected at mid-log phase. Subsequently, the cell was incubated at 30 °C under white
488 fluorescent lamps for 16-18 hr and selected by 25 µg/mL chloramphenicol and 5 µg/mL
489 spectinomycin on BG-11 solid media (1.5% w/v Bacto-agar).

490 Lipid analysis

491 For all organisms, FA analyses and for *O. tauri* further lipid analysis were achieved
492 accordingly to (Degraeve-Guilbault *et al.*, 2017). Organic solvents all contained
493 butylhydroxytoluene as an antioxidant (0.001%). For *N. benthamiana* frozen material (one leaf
494 broken into pieces) was preincubated in hot isopropanol (3ml, 75°C, 15 min, for PLD
495 inhibition), further extracted with CHCl₃ (1mL, Ultra-turax T25); Phase separation occurred
496 upon addition of NaCl 2.5% (2000g, 10 min). Pellet was re-extracted (3 mL CHCl₃: CH₃OH
497 2:1 v/v); Organic phases were washed twice with 0.25v of CH₃OH:H₂O (10:9 v/v). The lipid
498 extract was evaporated under a nitrogen stream and resuspended in CHCl₃:CH₃OH 2:1, v/v
499 (200 µL). Lipids were separated by HP-TLC and chloroform/methanol/ glacial acetic
500 acid/water (85:12:12:1 v/v/v/v). For *Synechocystis* (30mg DW, 3 mL CHCl₃:CH₃OH 2:1 v/v)
501 extraction was achieved using glass bead vortexing. HP-TLC developments were achieved in
502 the ADC2- chamber system (CAMAG) accordingly to (Degraeve-Guilbault *et al.*, 2017)
503 except for *Synechocystis* polar lipids (CHCl₃:CH₃OH/CH₃COOH/H₂O 85:12:12:1 v/v/v/v)
504 (Sallal *et al.*, 1990). Lipids were visualized and collected as previously described.

505 MS analyses of *O. tauri* glycerolipid species lipids was performed accordingly to the
506 method describe previously (Abida *et al.*, 2015). Purified lipids were introduced by direct
507 infusion (electrospray ionization-MS) into a trap-type mass spectrometer (LTQ-XL; Thermo
508 Scientific) and identified by comparison with standards. Lipids were identified by MS²
509 analysis with their precursor ion or by neutral loss analyses. Positional analysis of FA in
510 glycerolipids was achieved as previously described (Degraeve-Guilbault *et al.*, 2017).

511 Confocal microscopy

512 Live cell imaging was performed using a Leica SP5 confocal laser scanning microscopy
513 system (Leica, Wetzlar, Germany) equipped with Argon, DPSS, He-Ne lasers, hybrid
514 detectors, and 63x oil-immersion objective. *N. benthamiana* leave samples were transferred
515 between a glass slide and coverslip in a drop of water. Fluorescence was collected using
516 excitation /emission wavelengths of 488/490-540 nm for chlorophyll, 488/575- 610 nm for
517 YFP, and 561/710- 740 nm for m-cherry. Colocalization images were taken using sequential
518 scanning between frames. Experiments were performed using strictly identical confocal
519 acquisition parameters (*e.g.* laser power, gain, zoom factor, resolution, and emission
520 wavelengths reception), with detector settings optimized for low background and no pixel
521 saturation.

522 Photosynthesis measurement.

523 Measurements were made using a PhytoPAM (Heinz Walz GmbH, Germany).

524 *Light-response of photosystem II activity*

525 Rapid light-response curves (RLCs) of chlorophyll fluorescence of the cultures were
526 achieved accordingly (Serodio *et al.*, 2006). Briefly, the cultures were exposed to 12
527 increasing actinic light levels (10-s light steps of 100 μ E increase from 64 to 2064 μ E), and
528 the electron transport rates (ETR) were calculated on each step to draw RLCs. The following
529 parameters were extracted from the ETR-irradiance curve fitted to the experimental data: the
530 initial slope of the curve (α), the light-saturation parameters (I_k), and the maximum relative
531 electron transport rate (ETR_{max}).

532 *Photoinhibition experiment*

533 Optimal conditions for photosystem II inhibition and recovery were adapted from
534 (Campbell & Tyystjarvi, 2012). Cultures (50 mL, triplicate) were maintained under
535 fluorescent white light (low light: 30.4±1.0 µE, white light) without agitation at 20.2±0.2 °C
536 and moved to high light (117.6±4.9 µE, blue LED) for 45 min (photoinhibition);
537 photorecovery under initial condition was monitored for over 2 h. One mL sampling was used
538 to assess photosynthetic efficiency (quantum yield of photochemical energy conversion in
539 PSII; Y corresponds to Yield = dF/Fm).

540 *Sequences analyses*

541 DES domain-containing sequences were retrieved from genomic and transcriptomic data
542 from NCBI (Bioproject Accession: PRJNA304086 ID: 304086). Annotated ORF were
543 manually checked for the completion of Nt sequences in species from the class
544 Mamiellalophyceae; cTP were predicted from PredAlgo (Tardif et al., 2012); alignment of
545 Mamiellalophyceae homologous sequences was used to further determine putative cTP
546 assumed to correspond to the non-conserved Nt region (Snapgene trial version, Clustal
547 omega). These non-conserved regions were discarded for expression in *S. cerevisiae* and
548 *Synechocystis*. Codon-optimized sequences were obtained from Genewiz (Europe).

549 **Accession numbers**

550 [At]S8: Arabidopsis thaliana AEE80226.1, [Bo]6: Borago officinalis AKO69639.1,
551 [Ce] Caenorabditis elegans CAA94233.2, [Cr]4p Chlamydomonas reinhardtii AFJ74144.1,
552 [Ep]6 Echium plantagineum AAZ08559.1, [Eg]8 Euglena gracilis AA D45877.1, [Eh]
553 Emiliana huxleyi putative protein XP_005793257.1, [Gt] Guillardia theta CCMP2712
554 putative protein XP_005823787.1, [Ig]5/6 Isochrysis galbana ALE15224.1 and AHJ25674.1, [
555 Lb] Leishmania braziliensis putative protein XP_001569342.1, [Li]6 Lobosphaera incisa
556 ADB81955.1, [Ma]6 Mortierella alpine BAA85588.1, [Mp]6CoA Micromonas pusilla
557 XP_003056992.1, [Ms]6CoA Mantiella squamata CAQ30479.1, [No]6 Nannochloropsis
558 oculata, ADM86708.1, [Ot] Ostreococcus tauri CAL56435.1 (Δ6 acyl-CoA- DES Ot13.1),
559 CEF97803.1 (Ot05), CEF99426.1 (Ot10); CEG01739.1 (Ot15); CEF99964.1, (D5-DES),
560 CEF96519.1 (4ER), CEG00114.1, (D4p, Ot13.2), [Pt] Phaeodactylum tricorutum

561 XP_002185374.1 (putative protein), EEC45637.1 (D6-DES) EEC45594.1 (D5-DES), [Vc]
562 *Volvox carteri* XP_002953943.1, [SS] *Synechocystis* sp BAA18502.1, [Tp] *Thalassiosira*
563 *pseudonana*, XP_002289468.1 (putative protein) AAX14504.1 (S8); AAX14502.1 (8);
564 AAX14505.1 (6), XP_002297444.1 (4).

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569 Imaging was performed at the Bordeaux Imaging Center, member of the national
570 infrastructure France BioImaging.

571 **FIGURES LEGENDS**

572 **Figure 1. *O. tauri* front-end DES sequence features.** A. Phylogenetic tree of *O. tauri*
573 front-end DES and closest related homologs (Fast minimum evolution method). Species are
574 indicated in brackets, numbering refers to putative (*Italics*) or assessed DES D-
575 regiospecificity. S, sphingolipid-DES; p, plastidial DES; er, microsomal DES; 6CoA, acyl-
576 CoA Δ 6-DES. The three Δ 6/8-DES candidates are in bold and their label used in this paper in
577 brackets. Colors of nodes refer to the taxonomic groups: cyanobacteria (purple), eukaryotes
578 (gray), green algae (deep blue), eudicots (beige), cryptomonads (light blue), haptophytes
579 (light green), cryptomonads (yellow), euglenoids (pale pink) kinetoplastids (bright pink),
580 fungi (deep green) nematods (red). [At] *Arabidopsis thaliana*, [Bo] *Borago officinalis*, [Ce]
581 *Caenorabditis elegans*, [Cr] *Chlamydomonas reinhardtii*, [Ep]- *Echium plantagineum*, [Eg]
582 *Euglena gracilis*, [Eh] *Emiliana huxleyi*, [Gt] *Guillardia theta* CCMP2712, [Ig] *Isochrisis*
583 *galbana*, [Lb] *Leishmania braziliensis*, [Li] *Lobosphaera incisa*, [Ma] *Mortierella alpine*, [Mp]
584 *Micromonas pusilla*, [Ms] *Mantoniella squamata*, [No] *Nannochloropsis oculata*, [Ot]
585 *Ostreococcus tauri*, [Pt] *Phaeodactylum tricornutum*, [Vc] *Volvox carteri*, [SS] *Synechocystis*
586 sp PCC6803, [Tp] *Thalassiosira pseudonana*. B. Alignment of the acyl-CoA- Δ 6-DES and the
587 three Δ 6/8-DES candidates in the histidine-box regions. Histidine-box motifs are in blue
588 frames. Color highlighting is based on physical properties and conservation (clustal Omega):
589 positive (red), negative (purple), polar (green), hydrophobic (blue), aromatic (turquoise),
590 glutamine (orange), proline (yellow). Grey blocks highlight conservation only.

591 **Figure 2. Localization and activities of *O. tauri* acyl-CoA Δ 6-DES and Δ 6-DES**
592 **candidates in *N. benthamiana*.** **A.** Sub-cellular localisation of transiently overexpressed full-
593 length Ct-YFP-fused proteins. Images merged from Ot13-YFP and ER marker fluorescence
594 and from Ot05-YFP or Ot10-YFP and chlorophyll fluorescence. Experiments were repeated
595 at least twice. Images represent 100% of the observed cells (n). n=16 for Ot13-YFP, n =25 for
596 Ot05-YFP, n=21 for Ot10-YFP. Experiments were repeated at least twice. Images represent
597 100% of the observed cells (n). n=16 for Ot13-YFP (Acyl-CoA-D6-DES), n =25 for Ot05-
598 YFP, n=21 for Ot10-YFP. Bar, 10 μ m. **B.** FA-profiles of DES overexpressors. Means and
599 standard deviations of n independent experiments are plotted as histogram and the relative
600 production of ω -3 C18-PUFA (18:4n-3/18:3n-3) and ω -6 C18-PUFA (18:3n-6/18:2n-6) in
601 each experiment are shown in dot clouds. Dots corresponding to leaves used for the lipids
602 analysis showed in C are indicated by blue arrows. Control lines (p19) n=27, Ot13-OE n=17,
603 Ot10-OE n=21, Ot05-OE n=29. **C.** Relative production of ω -3 and ω -6 C18-PUFA in
604 glycerolipids. Cumulative ratio of pmol percent are plotted 18:4n-3/18:3n-3 yellow
605 bars, 18:3n-6/18:2n-6 red bars. On representative experiment out of two is shown (Fig. S7).

606 **Figure 3. Glycerolipid analysis of Δ desD *Synechocystis* PCC6803 Ot5-OE and Ot10-**
607 **OE.** Upper drawing indicates the respective role of desD and desB for the regulation of C18-
608 PUFA in *Synechocystis* PCC6803. C18-PUFA present at 34°C are highlighted in red, those
609 present at 24°C in blue. FA profile of glycerolipids at 34°C (**A, B, C, D**) and 24°C (**E, F, G,**
610 **H**). Means and standard deviations of three independent experiments are shown. MGDG and
611 DGDG displayed similar alterations and were cumulated (GL for galactolipids).

612 **Figure 4. Glycerolipid features of *O. tauri* Δ 6-DES-overexpressors.** **A.** Luminescence of
613 transgenic lines (Relative Luminescence Units from 200 μ l). Mean of triplicate and standard
614 deviations are shown. **B.** Cellular amount of ω -3-C18-PUFA **C.** Cellular amount of ω -6-C18-
615 PUFA. The labels v, 5, 10 and 13 correspond to lines transformed with empty vector, Ot5,
616 Ot10 and Ot13 respectively. **D.** Total glycerolipid FA profiles of lines selected for detailed
617 lipid analysis (Ot05-5, Ot10-5, Ot13-5). **B to D.** Means of triplicate independent experiments
618 and standard deviation are shown.

619 **Figure 5. Detailed Lipid analysis of *O. tauri* Δ 6-DES overexpressors.** **A to F.** Major
620 plastidic glycerolipids. **G, H.** Extrplastidic glycerolipids. For FA-profile analyses,

621 (A,C,E,F,G,H) means and standard deviations of three independent experiments are shown;
622 control line contains the empty vector. **B, D, F.** C18-PUFA molecular species analysis of
623 major plastidic lipids. Means and standard deviations of technical triplicate are shown.
624 Samples used for this analysis are independent from those used for GC-FID analysis; control
625 line is the wild-type (WT).

626 **Figure 6. Phosphate limitation and $\Delta 6$ -DES regulation in *O. tauri*.** **A-B.** Impact of
627 phosphate deprivation on C18-PUFA proportion (A) and desaturases transcript levels (B). **C.**
628 Photosynthetic inhibition responses of *O. tauri* $\Delta 6$ -DES-OE in phosphate- limited conditions.
629 Photosynthesis efficiency (Yield) was assessed under 30 $\mu\text{mol}/\text{m}^2/\text{s}$ (low light LL; Fig. S14)
630 before light intensity was increased for 45 min to 120 $\mu\text{mol}/\text{m}^2/\text{s}$ (high light HL:
631 photoinhibition) and put back to 30 $\mu\text{mol}/\text{m}^2/\text{s}$ (LL: recovery). Values are expressed as the
632 percentage of each culture's yield before photoinhibition (T_0). Means (\pm standard deviations)
633 of triplicates from independent cultures are shown. Cell density for control (i.e. empty vector
634 transgenic), Ot13-OE, Ot10-OE, Ot05-OE was in average, 48, 44, 32 and $48 \cdot 10^6$ cell/ml
635 respectively.

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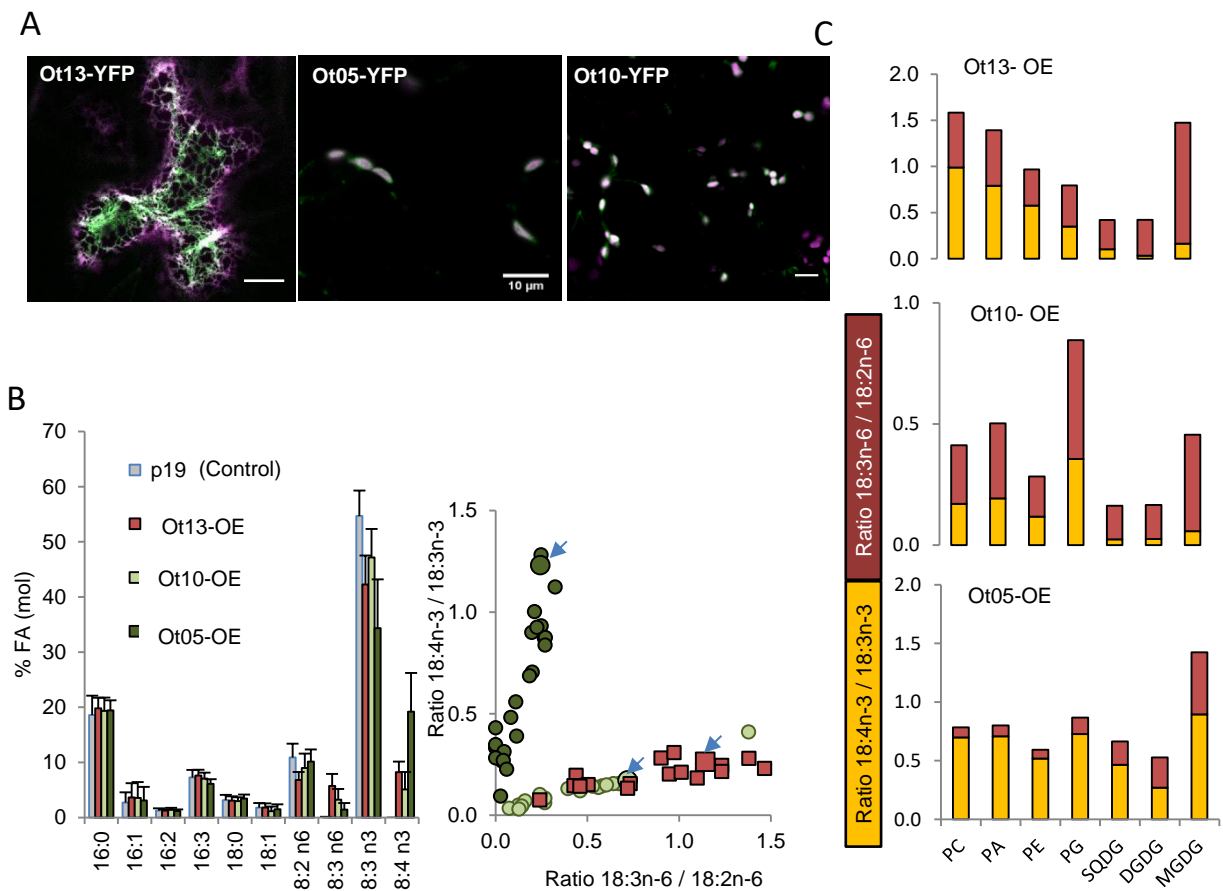


Figure 2. Localization and activities of *O. tauri* acyl-CoA $\Delta 6$ -DES and $\Delta 6$ -DES candidates in *N. benthamiana*. **A**. Sub-cellular localisation of transiently overexpressed full-length Ct-YFP-fused proteins. Images merged from YFP chlorophyll or ER-marker (Acyl-CoA- $\Delta 6$ -DES) fluorescences are shown. Experiments were repeated at least twice. Images represent 100% of the observed cells (n). n=16 for Ot13-YFP (Acyl-CoA- $\Delta 6$ -DES), n =25 for Ot05-YFP, n=21 for Ot10-YFP. Bar, 10 μ m. **B**. FA-profiles of DES overexpressors. Means and standard deviations of n independent experiments are plotted as histogram and the relative production of ω -3 C18-PUFA (18:4n-3/18:3n-3) and ω -6 C18-PUFA (18:3n-6/18:2n-6) in each experiment are shown in dot clouds. Dots corresponding to leaves used for the lipids analysis showed in C are indicated by blue arrows. Control lines (p19) n=27, Ot13-OE n=17, Ot10-OE n=21, Ot05-OE n=29. **C**. Relative production of ω -3 and ω -6 C18-PUFA in glycerolipids. Cumulative ratio of pmol percent are plotted 18:4n-3/18:3n-3 yellow bars, 18:3n-6/18:2n-6 red bars. On representative experiment out of two is shown (Fig. S7).

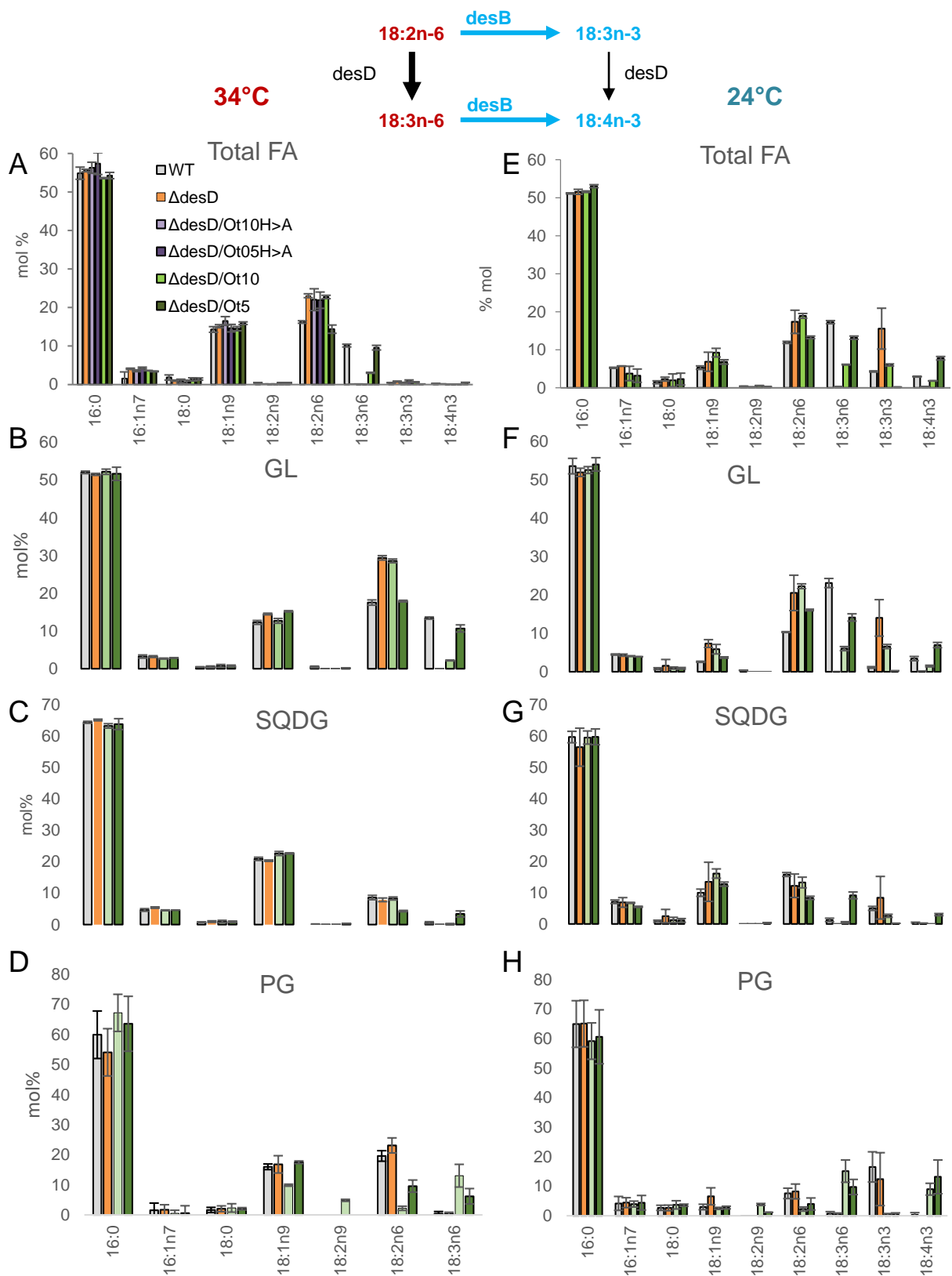


Figure 3. Glycerolipid analysis of $\Delta desD$ *Synechocystis* PCC6803 Ot5-OE and Ot10-OE. Upper drawing indicates the respective role of *desD* and *desB* for the regulation of C18-PUFA in *Synechocystis* PCC6803. C18-PUFA present at 34°C are highlighted in red, those present at 24°C in blue. FA profile of glycerolipids at 34°C (**A**, **B**, **C**, **D**) and 24°C (**E**, **F**, **G**, **H**). Means and standard deviations of three independent experiments are shown. MGDG and DGDG displayed similar alterations and were cumulated (GL for galactolipids).

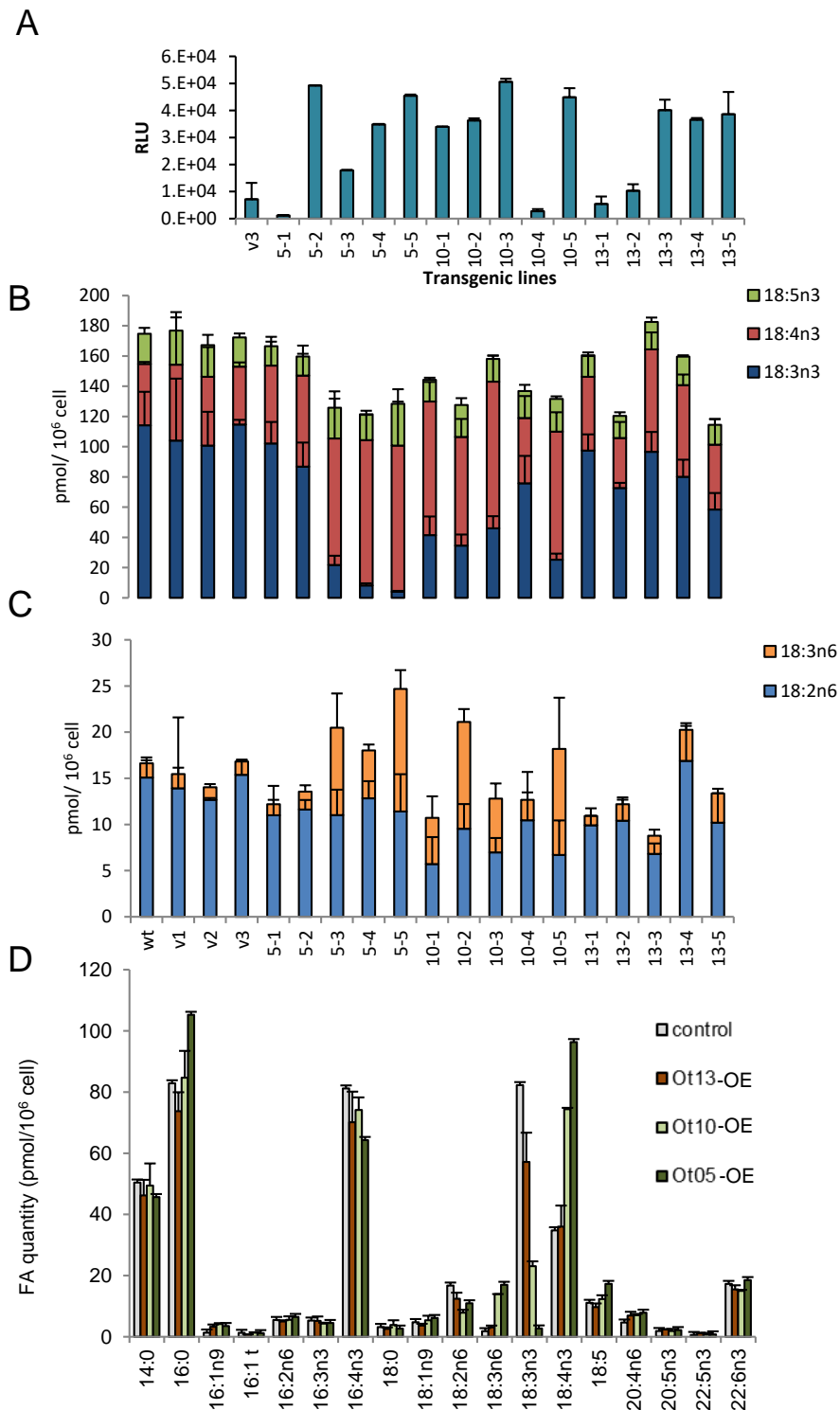


Figure 4. Glycerolipid features of *O. tauri* $\Delta 6$ -DES-overexpressors. **A**. Luminescence of transgenic lines (Relative Luminescence Units from 200 μ l). Mean of triplicate and standard deviations are shown. **B** cellular amount of ω -3-C18-PUFA **C**. C18-PUFA cellular amount of ω -6-C18-PUFA. The labels v, 5, 10 and 13 correspond to lines transformed with empty vector, Ot5, Ot10 and Ot13 respectively. **D**. Total glycerolipid FA profiles of lines selected for detailed lipid analysis (Ot05-5, Ot10-5, Ot13-5). B to D. Means of triplicate independent experiments and standard deviation are shown.

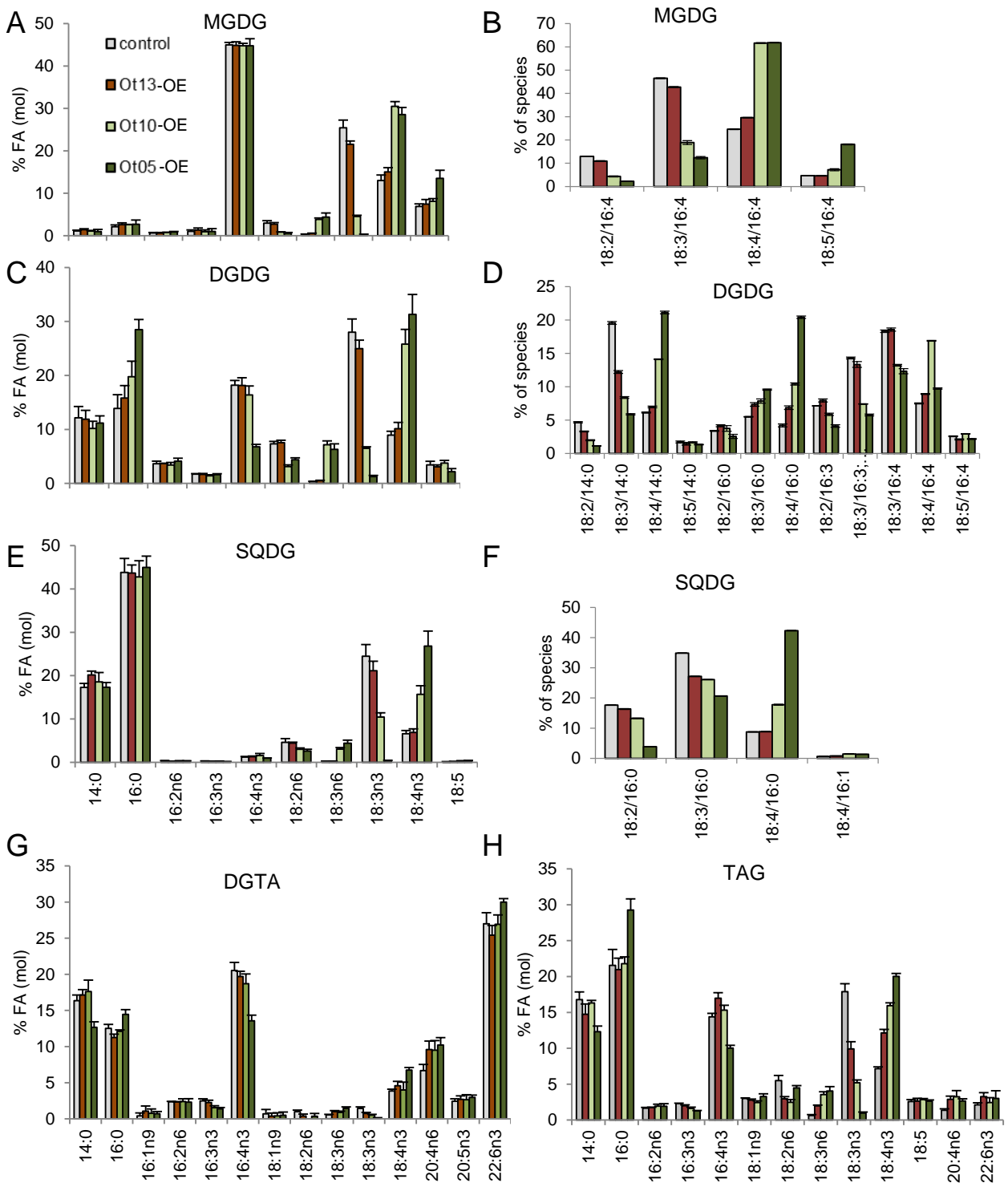


Figure 5. Detailed Lipid analysis of *O. tauri* $\Delta 6$ -DES overexpressors. **A to F**. Major plastidic glycerolipids. **G, H**. Extraplastidic glycerolipids. For FA-profile analyses, (A,C,E,F,G,H) means and standard deviations of three independent experiments are shown; control line contains the empty vector. **B, D, F**. C18-PUFA molecular species analysis of major plastidic lipids. Means and standard deviations of technical triplicate are shown. Samples used for this analysis are independent from those used for GC-FID analysis; control line is the wild-type (WT).

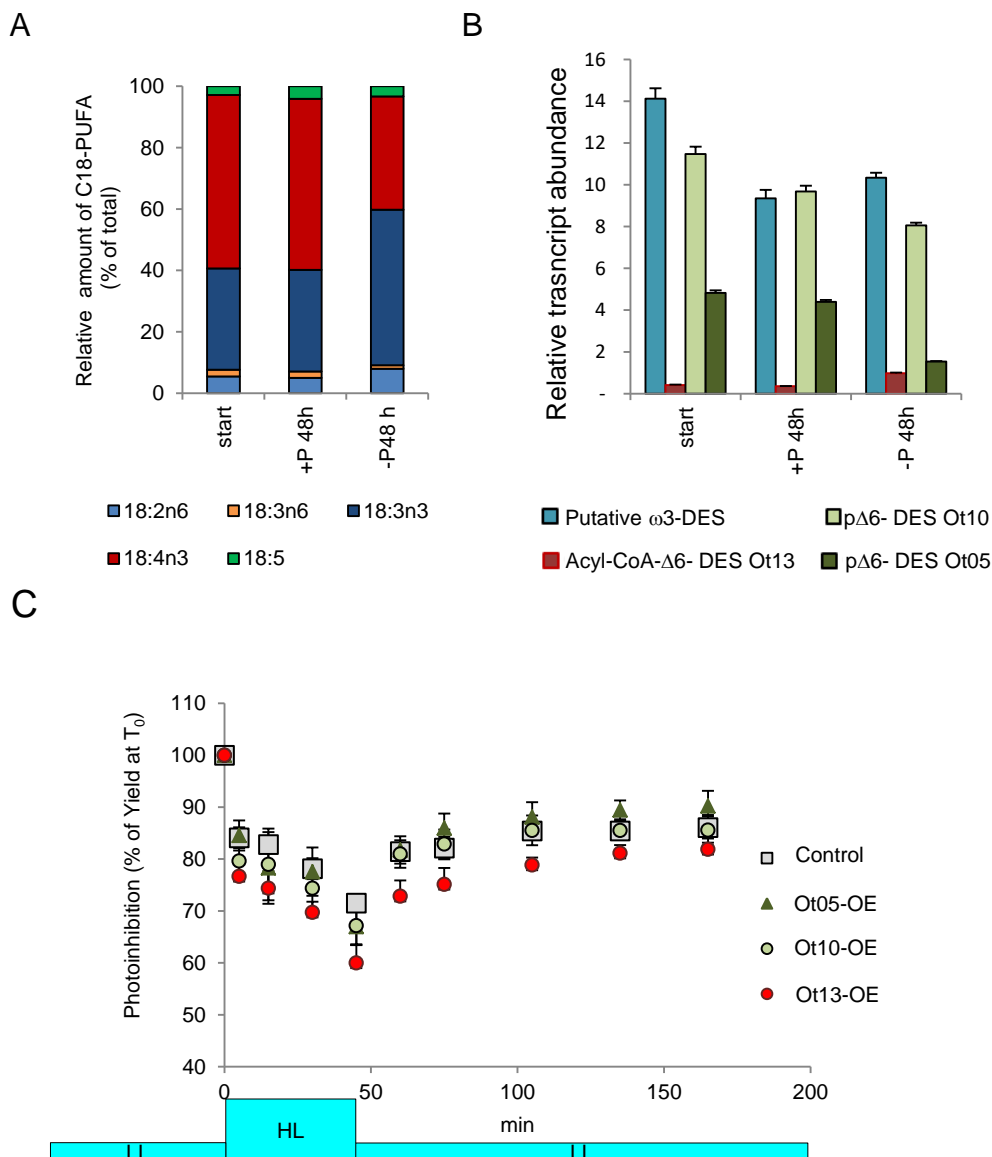


Figure 6. Phosphate limitation and Δ 6-DES regulation in *O. tauri*. **A-B**. Impact of phosphate deprivation on C18-PUFA proportion (A) and desaturases transcript levels (B). **C**. Photosynthetic inhibition responses of *O. tauri* Δ 6-DES-OE in phosphate-limited conditions. Photosynthesis efficiency (Yield) was assessed under 30 $\mu\text{mol}/\text{m}^2/\text{s}$ (low light LL; Fig. S14) before light intensity was increased for 45 min to 120 $\mu\text{mol}/\text{m}^2/\text{s}$ (high light HL: photoinhibition) and put back to 30 $\mu\text{mol}/\text{m}^2/\text{s}$ (LL: recovery). Values are expressed as the percentage of each culture's yield before photoinhibition (T_0). Means (\pm standard deviations) of triplicates from independent cultures are shown. Cell density for control (i.e. empty vector transgenic), Ot13-OE, Ot10-OE, Ot05-OE was in average, 48, 44, 32 and 48.10⁶ cell/ml respectively.

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