Plastidic Δ6 Fatty-Acid Desaturases with Distinctive Substrate Specificity Regulate the Pool of C18-PUFAs in the Ancestral Picoalga Ostreococcus tauri

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Eukaryotic Δ6-desaturases are microsomal enzymes that balance the synthesis of ω-3 and ω-6 C18-polyunsaturated fatty acids (C18-PUFAs) according to their specificity. In several microalgae, including Ostreococcus tauri, plastidic C18-PUFAs are strictly regulated by environmental cues suggesting an autonomous control of Δ6-desaturation of plastidic PUFAs. Here, we identified two putative front-end Δ6/Δ8-desaturases from O. tauri that, together with putative homologs, cluster apart from other characterized Δ6-desaturases. Both were plastid-located and unambiguously displayed a Δ6-desaturation activity when overexpressed in the heterologous hosts Nicotiana benthamiana and Synechocystis sp. PCC6803, as in the native host. Detailed lipid analyses of overexpressing lines unveiled distinctive ω-class specificities, and most interestingly pointed to the importance of the lipid head-group and the nonsubstrate acyl-chain for the desaturase efficiency. One desaturase displayed a broad specificity for plasticid lipids and a preference for ω-3 substrates, while the other was more selective for ω-6 substrates and for lipid classes including phosphatidylglycerol as well as the peculiar 16:4-galactolipid species occurring in the native host. Overexpression of both Δ6-desaturases in O. tauri prevented the regulation of C18-PUFA under phosphate deprivation and triggered glycerolipid fatty-acid remodeling, without causing any obvious alteration in growth or photosynthesis. Tracking fatty-acid modifications in eukaryotic hosts further suggested the export of plasticid lipids to extraplasticid compartments.

Marine microalgae synthesize peculiar polyunsaturated fatty acids (PUFAs) including hexadecatetraenoic acid (16:4Δ4,7,10,13), stearidonic acid (SDA, 18:4Δ6,9,12,15), and octapentaenoic acid (OPA, 18:5Δ4,7,10,13,16), as well as very-long-chain polyunsaturated fatty acids (VLC-PUFAs; Khozin-Goldberg et al., 2016; Jónasdóttir, 2019). Overexpression of both Δ6-desaturases in O. tauri prevented the regulation of C18-PUFA under phosphate deprivation and triggered glycerolipid fatty-acid remodeling, without causing any obvious alteration in growth or photosynthesis. Tracking fatty-acid modifications in eukaryotic hosts further suggested the export of plasticid lipids to extraplasticid compartments.
is specific to green microalgae (Chlorophyta; Lang et al., 2011). Several microalga classes from the Chromista kingdom, such as haptophytes and dinoflagellates, produce both OPA and DHA (Jónasdóttir, 2019). The only green microalgal species that also encompass OPA and DHA belong to the Prasinophyta phylum (Dunstan et al., 1992). Importantly, the Δ6-DES product SDA is abundantly present in Chromista (exception made of diatoms) and Prasinophyta, where it is a precursor for both OPA and DHA synthesis. In OPA-producing species, OPA is preferentially located in galactolipids (GaLs), whereas DHA is prevalent in extraplastidic lipids (Bell and Pond, 1996; Degraeve-Guilbault et al., 2017; Leblond et al., 2019). In diatoms, 20:5n-3, prevalent in GaLs, is thought to be imported from the endoplasmic reticulum (ER) to the chloroplast. All Δ6-DES characterized so far are microsomal enzymes. However, in contrast to 20:5n-3, C18-PUFAs, which are overwhelmingly present in GaLs of many Chromista species except for diatoms, do not require ER located elongases for their synthesis. Therefore, how the regulation of Δ6-DES and intercompartmental FA fluxes is involved in regulating the production and distribution of OPA and DHA in microalgae species remains an important and unanswered question.

*Ostreococcus tauri* (class Mamiellophyceae, order Mamiellales) is an ancestral green picoalga that emerged early after the divergence between Chlorophyta and Streptophyta (land plants; Leliaert et al., 2012). *O. tauri* has the most minimal genomic and cellular organization (Derelle et al., 2006). This coccoid cell is smaller than 2 μm (a picoeukaryote), lacks a cell wall, flagella, or an obvious sexual life (Grimsley et al., 2010). However, it displays a large panel of PUFA, including hexadecatetraenoic acid, ALA, SDA, OPA, and DHA as major PUFAs (Wagner et al., 2010; Degraeve-Guilbault et al., 2017). *O. tauri* glycerolipids (GLs) characterization unveiled an exceptionally clear-cut allocation of PUFA in membranes: C18-PUFAs are prevalent in plastidic lipids, with OPA being restricted to GaL, whereas VLC-PUFA are exclusively found in extraplastidic GLs (Degraeve-Guilbault et al., 2017). Many microalgae, including *Chlamydomonas reinhardtii*, *Ostreococcus*. spp., and related genera are devoid of phosphatidylcholine (PC). For *O. tauri*, the major extraplastidic GLs identified correspond to the betaine lipid diacylglycerol-hydroxymethyl-trimethyl-β-Ala (DGTA) and the peculiar phosphosulfolipid phosphatidyltrimethyl-propanethiol (PDPT), both of which are typical of Chromista species. Interestingly, we uncovered that the plastidic C18-PUFA pool was strictly regulated by nutrient availability: under phosphate or nitrogen deprivation, ALA content was increased at the expense of SDA in plastidic GLs, the acyl-CoA pool, and triacylglycerols (TAGs), but not in extraplastidic GLs (Degraeve-Guilbault et al., 2017). This previous result strongly suggested that nutrient starvation specifically inhibited Δ6-DES of plastidic lipids; however, so far, the Δ6-DES characterized from *O. tauri* and related species was demonstrated to be microsomal and to have the peculiarity of using acyl-CoA as substrates (Domergue et al., 2005; Hoffmann et al., 2008; Petrie et al., 2010a, 2010b; Vaezi et al., 2013). We therefore reasoned that the specific regulation of plastidic C18-PUFA in Mamiellales likely involves uncharacterized plastidic Δ6-DES, rather than the transfer of Δ6-DES products from the acyl-CoA pool to the chloroplast.

### RESULTS

*O. tauri* Fatty Acid Desaturase Sequences Retrieval and Analysis

Thirteen canonical DES sequences were retrieved from genomic and transcriptomic databases (National Center for Biotechnology Information [NCBI] databases; Supplemental Table S1). All sequences were manually checked upstream of the predicted start codon, especially to assess the completion of the protein N-terminal (Nt) part; when required, extended Nt were validated by complement DNA (cDNA) amplification (see “Materials and Methods”; Supplemental Table S2). Except for an acyl-CoA-Δ6-DES (Ot13, according to the genomic accession) and an unknown DES barely related to sphingolipid Δ3/Δ4-DES, complete sequences (using the prediction software PredAlgo; Tardif et al., 2012) were all predicted to contain a chloroplastic target-peptide (cTP). Among the seven front-end DESs, three uncharacterized Δ6/Δ8 FA-DESs retained our attention (Fig. 1; Supplemental Table S1). All three reference sequences from the *O. tauri* strain RCC4221 were completed at the Nt to include a predicted cTP (see

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Figure 1. Phylogenetic tree of O. tauri front-end DES and homologs in other species. The tree was constructed from the tool COBALT (NCBI) using the accessions indicated below (Fast minimum evolution method, Maximum sequence difference 0.85, and Grishin’s distance). Species are indicated in brackets; numbering refers to putative (in italics) or using the accessions indicated below (Fast minimum evolution method, Maximum sequence difference 0.85, and Grishin’s distance). Species are indicated in brackets; numbering refers to putative (in italics) or

Materials and Methods”; Supplemental Figs. S1–S3; Supplemental Table S1). One 6-DES candidate (Ot15) clustered with acyl-lipid Δ6/Δ8-DES and was closest to the diatom Thalassiosira pseudonana Δ8-sphingolipid-DES (Fig. 1; Tonon et al., 2005). The two other candidates (Ot05 and Ot10) were closely related (49.8% identity, 71.6% similarity), and together with putative homologs, formed a cluster apart from the typical acyl-CoA Δ6-DES specific to Mamiellophyceae species and acyl-lipid Δ6/Δ8-DES from plants, fungi, and worms (Supplemental Fig. S1B). Each of these two candidates had one putative homolog in the Mamiellophyceae species, with the exception of Bathycoccus prasinos, for which the only homolog was more closely related to Ot05 (Supplemental Figs. S2 and S3). The His-boxes (His-Box), typical of the DES domain, displayed consensus motifs contrasting with those from acyl-CoA-Δ6-DES (Table 1; Supplemental Figs. S2 and S3; Sayanova et al., 1997; López Alonso et al., 2003). A further conserved region occurred upstream of the third His-Box and was also divergent between the Δ6-DES candidates and the acyl-CoA-Δ6-DES. More distantly related putative homologs occurred in microalgae arising from secondary endosymbiosis, i.e. species from the Chromista and Euglenozoa supergroup (Supplemental Fig. S4). All homologous sequences, which were annotated as putative proteins, displayed the typical Nt-fused-Cyt-b5 domain found in front-end DES, and distinctive His-Box consensus motifs emerged from their alignment (Table 1; Supplemental Fig. S4).

The O. tauri acyl-CoA-Δ6-DES (Ot13) was previously characterized in yeast, and extensively used for the reconstruction of VLC-PUFA pathways 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, so Ot13 was chosen as a supplemental reference to achieve the functional characterization of the two closely related Δ6-DES candidates.

Δ6-DES-Candidate Localization and Activities in Heterologous Hosts

Full-length or codon-optimized Nt-truncated (for removing the putative cTP) open reading frames (ORFs) were expressed in Saccharomyces cerevisiae (Supplemental Fig. S5A). When 18:3n-3 was supplied, none of the candidates, either full length or Nt-truncated, displayed Δ6-activity. However, expression of the Ot13 (the acyl-CoA Δ6-DES) produced significant amounts of 18:4n-3. Neither the supply of Δ3-, Δ5-, or Δ8-substrates, nor the coexpression with the Ot13, resulted in the appearance of novel FAs; this clearly indicated that neither Ot05 nor Ot10 were active in yeast (Supplemental Fig. S5B). Overexpression of full-length C-terminal–fused yellow fluorescent protein (Ct-YFP) in Nicotiana benthamiana unveiled that both Ot05 and Ot10 exclusively localized at plastids, while Ot13 was at the ER (Fig. 2A). Furthermore, overexpression of the full-length ORFs in N. benthamiana led to the synthesis of 18:3n-6 and 18:4n-3 from the endogenous substrates 18:3n-3 and 18:2n-6, indicating that both Ot05 and Ot10 displayed Δ6-DES activity in this host.
Table 1. Consensus motifs of the Hox boxes of Δ6/Δ8-DES candidates

Motifs were retrieved from alignment from homologs of Ot13 (acyl-CoA Δ6-DES), Ot05, Ot10, or Ot15 in Mamiellophyceae (Mam) species and from Ot05 and Ot10 homologs of a distantly related species (OP, other species). Accession for homologs of Ot13 (Supplemental Fig. S4), of Ot05 (Supplemental Fig. S2), of Ot10 (Supplemental Fig. S3), of Ot15, XP_001417552 (Oli), XP_001063519 (Mpi), XP_00250630 (Mc), and XP_007515499 (Bp).

| Motif       | H-Box1            | H-Box2            | H-Box3            |
|-------------|-------------------|-------------------|-------------------|
| Ot13 Mam    | QHEGGH/NSL        | WNNXH/NSL/HHH     | QVHHH/LFHDMP      |
| Ot05 Mam    | GHDDYV/HRGC       | WWYXQ/KS/S/MHH    | QLEHH/LFP         |
| Ot10 OP     | AHDDYV/1/THGRG    | WWVQ/SKHN/MMH     | QLV/LH/LFP        |
| Ot10 OP     | XHDYXHGrX         | WXXKH/NXH         | QLEH/LFP          |
| Ot15 Mam    | GHDAGHXXIT        | WXXTHV/NVH       | QFQ/VIHHHLC       |

(Fig. 2B). Interestingly, disrupting the heme-binding capacity of Cyt-b5 by H > A mutation in the HPGG motif abolished the activity of Ot05 and Ot10 (Supplemental Fig. S6A; Sayanova et al., 1999). Note that Ct-YFPs were also active in N. benthamiana (Supplemental Fig. S6B). Altogether, these results unambiguously demonstrated that both Ot05 and Ot10 are plastidic Δ6-DES (pΔ6-DES).

Significant differences emerged from the bulk of the experiments (Fig. 2, B and C). First, the Δ6-DES product 18:3n-6 was highest in Ot13-OE and lowest in Ot05-OE. The proportion of 18:2n-6 remained stable in pΔ6-DES-OE, suggesting that 18:2n-6 was a nonlimiting substrate for the production of 18:3n-6 in Ot05-OE and Ot10-OE. In contrast, 18:3n-3 was significantly reduced in all overexpressors and to a greater extent in Ot05-OE (Fig. 2B). In parallel, the proportion of 18:4n-3 was significantly higher in Ot05-OE compared to Ot10-OE and Ot13-OE. These observations strongly suggested that the higher production of 18:4n-3 in Ot05-OE mostly resulted from the preference of Ot05 for ω-3 compared to ω-6 substrates, whereas Ot13 and Ot10 both displayed a preference for ω-6 substrates. Considering the ω-class substrate conversion efficiency in each sample, Ot13-OE and Ot10-OE displayed a similar trend, with the conversion of ω-6 C18-PUFA being approximately three times higher than that of ω-3; conversely, ω-3 substrate conversion was approximately twice that of ω-6 in Ot05-OE (Fig. 2C).

The relative impact on GL classes of each Δ6-DES-OE, expressed as a conversion percent for each lipid, was analyzed from two independent experiments (Fig. 2D, Supplemental Fig. S7). The global lipid profile appeared unchanged in overexpressor lines (Supplemental Fig. S7A). In Ot13-OE, extraplastidic phospholipids including phosphatidylcholine (PC), phosphatidylycerine together with phosphatidic acid and phosphatidylethanolamine, were more impacted compared to plastidic GLs (Fig. 2D; Supplemental Fig. S7, B and C): the ω-6 conversion was nevertheless importantly increased in monogalactosyl diacylglycerol (MGDG) and a relatively high conversion rate was observed for phosphatidylglycerol (PG). Conversely, although extraplastidic GLs were obviously impacted in the pΔ6-DES-OE, the most altered lipids were MGDG in Ot05-OE and PG in Ot10-OE. Note that in Ot10-OE, the PG ω-3 and ω-6 indexes were both the highest. Finally, the overall impact on sulfoquinovosyl diacylglycerol (SQDG) was slightly more pronounced for Ot05-OE by considering the maximal index in Ot05-transgenics compared to that in Ot13 and Ot10 transgenics.

To further clarify the substrate specificity of pΔ6-DES, the cyanobacterium Synechocystis sp. PCC 6803 was used. This organism not only encompasses the eukaryotic classes of plastidic lipids as major GLs but also allows transgene expression from a similar genomic environment (homologous recombination; Williams, 1988). Synechocystis sp. PCC 6803 has one Δ6-DES (desD) and one ω-3-DES (desB). In wild type, the selectivity of DesD for GaLs is reflected by the exclusive distribution of 18:3n-6 in GaLs (Fig. 3; Wada and Murata, 1990). The transcription of desB is induced at temperatures below 30°C and results in 18:3n-3 accumulation in PG and SQDG, and of 18:4n-3 in GaLs. Note that all GL species are sn-1/sn-2 18: X/16:0 combinations.

Expressing either Ot05 or Ot10 in ΔdesD cells of Synechocystis at 34°C (no endogenous ω-3-DES activity) resulted in 18:3n-6 production at the expense of 18:2n-6 (Fig. 3A). As for N. benthamiana, Overexpressor of an H > A mutated version of the Δ6-DES resulted in the absence of Δ6 products, indicating that integrity of the HPGG motif in the Cyt-b5 domain was required (Fig. 3A). The Ot05-OE restored the 18:3n-6 wild-type production, whereas the Ot10-OE resulted in a much lower production, indicating a higher ω-6 activity of Ot05 compared to Ot10 in this host. Most interestingly, the accumulation of 18:3n-6 occurred not only in GaLs of both pΔ6-DES-OE, but also in PG as well as in SQDG for Ot05-OE, contrasting with the wild-type features (Fig. 3, B–D). Noteworthy is that the amount of 18:3n-6-PG was twice as high for Ot05-OE than Ot10-OE (isomer of 18:2), likely corresponding to the Δ6 desaturation product of 18:1n-9, as seen in Figure 3D.

For all lines, the induction of DesB at low temperature resulted in the synthesis of 18:3n-3 in all lipid classes. In the wild type, the increase of 18:3n-6 at the expense of 18:2n-6 reflected that the Δ6-DES was prevalent over the ω-3-desaturation of 18:2n-6, and further, that the ω-3-desaturation of 18:3n-6 limited the 18:4n-3 synthesis (Fig. 3E). Overexpression of O. tauri pΔ6-DES in ΔdesD restored the production of 18:4n-3, although to a lower extent than in Ot10-OE compared to the wild type, and...
in Ot05-OE (Fig. 3E). Compared to the wild type, the amount of 18:3n-6 was decreased to a greater extent in Ot05-OE and that of 18:3n-3 was depleted, suggesting that the 18:2n-6, 18:3n-3, 18:4n-3 route prevailed over the 18:2n-6, 18:3n-6, 18:4n-3 route. These changes were mostly reliant upon variations occurring in the GaLs that are quantitatively prevalent. However, the synthesis of 18:4n-3 was further unambiguously detected in PG for pD6-DES-OE, mostly at the expense of 18:3n-3, and in SQDG for Ot05-OE (3% of SQDG species; Fig. 3, F–H).

Altogether, these results indicated that: (1) Ot05 displays an overall preference for ω-3, in agreement with the conclusion drawn from N. benthamiana; (2) O. tauri pΔ6-DES accepted both 18:X/16:0 GaLs as substrates, with Ot10 being less efficient than Ot05; (3) PG was impacted to a greater extent in Ot10-OE compared to other lipids; and (4) Ot05 accepted SQDG as substrate (Fig. 3, D and H).

**Δ6-DES Overexpressor in O. tauri**

To gain insight into the regulation of C18-PUFA pool by Δ6-DES in the native host, O. tauri overexpressors of each Δ6-DES were created using the pOtOXLuc vector, in which the high-affinity-phosphate-transporter promoter drives the overexpression while the luciferase reporter gene, under the CCA1 promoter, allows ready screening of the best transgenics (Moulager et al., 2010).
Screening and Selection of Δ6-DES Transgenic Lines

Cultures were grown under low phosphate conditions to allow full activation of the high-affinity-phosphate-transporter promoter. These conditions were previously suspected to prevent Δ6-DES of C18-PUFAs, and were therefore ideal for the screening of Δ6-DES OE (Degraeve-Guilbault et al., 2017). Transgenics of each of the Δ6-DES were retrieved according to their luminescence level. Five transgenics were selected to ascertain their FA phenotype and determine the level of Δ6-DES gene expression (Fig. 4; Supplemental Figs. S8 and S9). The relative content of C18-PUFA of these transgenics varied accordingly to the luminescence and transgene expression levels. For similar luminescent levels, the relative amount of ω-3 C18-PUFA varied to a greater extent in Ot05-transgenics compared to Ot10 transgenics, whereas that of ω-6-C18-PUFA was similarly impacted in Ot05 and Ot10 transgenics; these variations, although significant, were far more modest for most luminescent Ot13 transgenics (Fig. 4C; Supplemental Fig. S9). Considering the averages of the best transgenics for each desaturase, it clearly appeared that pΔ6-DES OE importantly impacted ω-3-C18-PUFA, including an increase in 18:5n-3 for the best Ot05 OE (Supplemental Fig. S9). Although the impact of Ot05 OE was the most pronounced, it was striking that the impact of Ot10 OE was much greater than in heterologous hosts. Furthermore, considering the average values from the best transgenics, the proportion of 16:4n-3 was most significantly decreased whereas that of 16:0 was increased in Ot05 lines (Supplemental Figs. S8 and S9).

One transgenics out of five that was representative of each Δ6-DES OE was selected (lines Ot13-5, Ot10-5, and Ot05-5), taking care that expression levels of Δ6-DES were similar, and further detailed lipid analyses were achieved (Supplemental Fig. S8C). These lines are referred to as Ot13-OE, Ot10-OE, and Ot05-OE.

![Diagram](image-url)
hereafter (Fig. 4, D and E). Note that no growth defect was detected in the best D6-DES-OEs (Fig. S10).

Lipidic Features of Selected D6-DES OEs

The relative amount of TAG was significantly increased in Ot13-OE and Ot05-OE, while MGDG was significantly decreased in all overexpressors (Fig. 4E). Alterations of C18-PUFA were observed in each of the major plastidic lipid classes (Fig. 5). For ω-6 C18-PUFA, changes in GaLs were similar between Ot10-OE and Ot05-OE. For ω-3 C18-PUFA, changes in GaLs were more pronounced in Ot05-OE, with a higher reduction of 18:3n-3 and an increase of the Δ3-desaturation product 18:5n-3 in MGDG (18:5/16:4-MGDG; Fig. 5, A, B, and D). A drastic reduction of 16:4n-3, paralleled by an important rise in 16:0, was unambiguously detected in DGDG for Ot05-OE (Fig. 5C). Overall, SQDG C18-PUFA were also more impacted in Ot05-OE (Fig. 5C).

Mass spectrometry (MS) analyses allowed us to compare variations between D6-OE for a given molecular species; they highlighted that the molecular species 18:4/14:0 and 18:4/16:0 in DGDG and SQDG were increased to a greater extent in Ot05-OE than in Ot10-OE, whereas the variations of 18:X/16:4 18:X/16:3 GaL species were closer to one another except for 18:4/16:4-DGDG (Fig. 5, E and F). Indeed, 18:4/16:4-DGDG was only increased in Ot10-OE, whereas 18:3/16:4-DGDG was decreased in both Ot10-OE and Ot05-OE, but not in Ot13-OE (Fig. 5E). Altogether, these variations might explain the lower relative amount of 16:4n-3 recorded from the gas chromatography with flame ionization detection (GC-FID) analysis of Ot05-OE DGDG (Fig. 5B).

Minor alterations were detected in DGTA, which is the prevailing extraplastidic structural lipid class under phosphate-limited conditions (Degraeve-Guilbault et al., 2017). The proportion of 20:4n-6 was similarly increased in the three Δ6-DES-OEs, while subtle changes were further detected in Ot05-OE, among which the most interesting consisted of a higher amount of 18:4n-3 and a lower amount of 16:4n-3 (Fig. 5G). These latter changes might be related to the variations observed for several DGTA species in Ot05-OE; these species include 32:4 (14:0/18:4), which is increased, as well as 30:4 (14:0/16:4), 36:8 (putatively 20:4/16:4), and 38:10-DGTA (22:6/16:4). All of these are decreased to a greater extent in Ot05 compared to other lines (Supplemental Fig. S11).

Finally, TAG FA-profile and molecular species were importantly impacted by the overexpression of each of the three Δ6-DES lines (Fig. 5H; Supplemental Figs. S12 and S13). Noteworthy, regarding Ot13-OE, is the clear alteration of the ratio of Δ6-DES-substrates/Δ6-DES-products in TAGs, that contrasted with the minor alterations detected in structural lipids (Fig. 5H). For Ot05-OE, the level of 16:4n-3 was also significantly reduced while that of 16:0 was increased, recalling the changes occurring in DGTA and DGDG.

Despite the complexity of mass-to-charge ratio (m/z) TAG species, distinctive features emerged from MS analyses. Overall, the most pronounced changes in all Δ6-OE were related to m/z species containing Δ6-substrates and products that corresponded to the major species in wild type: the 18:3-TAG species 48:3 and 50:3 were the most reduced, whereas the 18:4-TAG species 48:4, 50:4, and 50:8 were the most increased (Supplemental Figs. S12 and S13). For Ot05-OE, the species combining 22:6 and 18:4 (54:10, 56:6) were
among the highest values. The species 50:10 (16:4/16:3/18:3 and possibly 16:4/16:4/18:2) and 50:11 (16:4/16:4/18:3) were the second most reduced species, with the lowest signal for Ot05-OE. Looking for TAG species that might be related to the specific variation of C16 FA in Ot05-OE (Fig. 5H), 16:4/m/z species accounting for the reduction of 16:4 possibly encompassed 48:7 (which includes 16:4/14:0/18:3), 50:6 (which includes 16:4/16:0/18:2), and 50:7 (which includes 16:4/16:0/18:3), while 16:0/m/z species accounting for 16:0 increase might be 54:10 (which includes 16:4/16:0/22:6), 56:9 (which includes 18:4/16:0/22:5), and 56:10 (18:4/16:0/22:6). All of these species encompassed an unsaturated FA at the sn-2 position, pointing to the possibility that changes in TAG species were related to the remodeling of glycerol moieties of plastidic structural lipids (Degraeve-Guilbault et al., 2017).

Physiological Relevance of \( \Delta 6 \)-DES Regulation

Transcriptional regulation of desaturases is known to occur in response to environmental cues. We therefore assessed transcript levels of desaturases involved in the regulation of the C18-PUFA pool by phosphate availability, including the putative \( \omega 3 \)-DES (Kotajima et al., 2014). Consistent with our previous report, the proportion of 18:3n-3 was increased by approximately one-half after the transfer of cells to phosphate-depleted medium (Fig. 6A). By that time, the transcript level of Ot05 was decreased by 60%, the transcript levels of Ot10 and of the putative \( \omega 3 \)-DES remained stable, and transcript levels of the acyl-CoA DES were increased (Fig. 6B). This result indicated that a decrease in Ot05 activity through transcriptional repression resulted in lowering of the 18:4n-3/18:3n-3 ratio under phosphate deprivation.

Thylakoid membrane PUFA composition is known to influence photosynthetic processes (Allakhverdiev et al., 2009). With the aim of assessing a possible impact of C18-PUFA alterations on photosynthesis, photosynthetic parameters and photoinhibition responses were investigated in the \( \Delta 6 \)-DES-OE Ot05 and Ot10. Photosynthetic parameters were recorded from two
independent batches at stationary stage. Interexperimental variations were high and the slight differences observed in one of the experiments appeared to be not reproducible (Table 2; Supplemental Table S4). We therefore concluded that the effect of the transgene overexpression, if any, was not prevalent over the differences related to batch culture conditions, i.e., self-shading slight differences of progression into stationary phase. Photoinhibition and recovery responses of \(\delta6\)-DES OE were also similar to control lines. Accordingly, we concluded that no obvious alterations regarding the photosynthesis features investigated could be detected in \(\delta6\)-DES-OE in our conditions. Further detailed investigations will be required to possibly unveil photosynthesis defects and/or compensatory mechanisms in these lines.

**DISCUSSION**

FA remodeling of structural lipids in response to chilling in plants and cyanobacteria involves the regulation of C18-PUFA desaturation (Los et al., 2013); downstream synthesis of \(\omega-3\) and \(\omega-6\) VLC-PUFA in animals, fungi, and microalgae also involves the fine-tuning of C18-PUFA, notably by \(\delta6\)-DES. On the other hand, all front-end \(\delta6\)-DES studied so far are demonstrated to be, or assumed to be, located at the ER (Meesapyodsuk and Qiu, 2012). By unveiling the first plastidic \(\delta6\)-DES in the ancestral green picoalga \(O.\) *tauri*, our work points out the autonomous control of plastidic C18-PUFA in microalgae. The entangled substrate features instructing us on the activity of these two novel \(\delta6\)-DES, the possible PUFA fluxes unveiled by their overexpression, and the physiological significance of \(\delta6\)-DES, are discussed here.

**Substrate Specificity of \(O.\) *tauri* \(\delta6\)-DES**

DES specificity relies on intricate substrate features, including the acyl-chain position, length, and unsaturation level, as well as the acyl-carrier nature (Heilmann et al., 2004a; Li et al., 2016a). Although the primary importance of the His-Box and adjacent regions for front-end desaturase activity and substrate specificity could be demonstrated, neither the exact molecular features underlying DES (regio)specificity nor the hierarchical importance of these substrate features have yet been clearly identified (Song et al., 2014; Li et al., 2016a; Watanabe et al., 2016). Furthermore, assaying plant DES activity in *S. cerevisiae*, which lacks plastidic lipid classes, might have introduced some bias by favoring the identification of microsomal desaturases and/or by hampering the proper characterization of plastidic substrates of desaturases (Heilmann et al., 2004b).

In this work, four different hosts were used to characterize \(\delta6\)-DES substrate specificity. Lipid changes occurring in each of these organisms reflect a steady state arising from both desaturation and overall FA fluxes. Nevertheless, comparison of lipid features triggered by the overexpression of each \(\delta6\)-DES in a given host, and of the same \(\delta6\)-DES in different hosts, allowed us to gain insight into \(\delta6\)-DES substrate specificity. In 16:3-plants (Arabidopsis \(\text{[Arabidopsis thaliana]}\) and *N. benthamiana*), the Kennedy pathway contributes to the synthesis of plastidic lipid yielding \(\text{sn-1/sn-2}\) 18:3/18:3 species in addition to the 18:3/16:3-lipid species that arise from de novo plastidic synthesis (Browse et al., 1986b). In *O. tauri*, as in *C. reinhardtii* and cyanobacteria, plastidic lipids correspond to \(\text{sn-1/sn-2}\) 18:X/16:X species while the major extraplastidic lipid species are di-VLC-PUFA in DGTA (22:6/22:6) and saturated FA/16:4, and VLC-PUFA/16:4 in both DGTA and PDPT (Degraeve-Guilbault et al., 2017). These distinctive positional signatures strongly suggest that, in *O. tauri*, plastidic lipid synthesis is independent of ER synthesis (Ohlrogge and Browse, 1995). On the other hand, acyl-lipid remodeling of plastidic lipids is assumed to be absent in *Synechocystis* species, for which no acyl-turnover was ever reported. Concerning microalgae, MGDG has been proposed, yet not clearly proven.

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**Figure 6.** Phosphate limitation and \(\delta6\)-DES transcriptional regulation in *O. tauri*. A, Impact of phosphate deprivation on C18-PUFA proportion. B, Transcript levels of desaturases.
to be a platform of FA exchange supporting the incorporation of plastidic FA into TAGs in *C. reinhardtii* (Li et al., 2012; Kim et al., 2018). We have based the interpretation of our results on this knowledge.

### Head-Group Specificity

As major changes occurred in GaLs independently of the host, it can reasonably be concluded that at least MGDG is a substrate of both Ot05 and Ot10. Most interestingly, Ot05 OE in *Synechocystis* PCC 6803 triggered the accumulation of α6-DES products in SQDG; SQDG was also unambiguously more impacted in *Synechocystis* PCC 6803 trig-
between the DES and the acyl-carrier was indeed fundamental to orient the acyl-chain in the catalytic tunnel of the enzyme (Wang et al., 2015).

The O. tauri acyl-CoA-Δ6-DES has been reported to efficiently produce Δ6-DES products in the heterologous host, but it has never been characterized in the native host. We confirmed that O. tauri acyl-CoA-Δ6-DES OE in N. benthamiana resulted in the accumulation of Δ6-DES products in all phospholipids; its overexpression in O. tauri moderately impacted structural GLs, but led to important accumulation of Δ6-DES products in TAGs. These results suggested that over-supplied Δ6-DES products from the acyl-CoA pool are preferentially incorporated into TAGs in the native host, whereas in N. benthamiana leaves, they are preferentially incorporated into PC, and then in part, likely transferred to MGDG (see below).

\(\omega-3/\omega-6\) and 16:4-GL Species Selectivity

For all host organisms in this study, the impact of Ot05-OE on \(\omega-3\)-C18-PUFA was more pronounced compared to that of Ot10, indicating a preference of Ot05 for \(\omega-3\)-substrates. Conversely, Ot10 appeared globally more selective for \(\omega-6\) substrates. Nevertheless, compared to other organisms, the overexpression of Ot10 in O. tauri impacted the amount of \(\omega-3\)-C18-PUFA to a greater extent, especially for 16:4-Gal species. Considereing that Ot10 could display a higher \(\omega-6\) desaturation activity in the native host, the \(\omega-3\)-desaturation of the overproduced 18:3n-6 in Ot10-OE might be involved in the rise of 18:4n-3. However, one would expect a higher accumulation of 18:3n-6 in Ot10-OE compared to Ot05-OE if only this route was used, which is not the case. Indeed, the endogenous \(\omega-3\)-desaturase and the overexpressed Δ6-DES compete for 18:2n-6; consequently, if 18:3n-6 production prevailed over that of 18:3n-3 (i.e. Δ6-DES activity higher than \(\omega-3\) activity), one would expect that the conversion of 18:3n-6 to 18:4n-3 would be limited and would result in the higher proportion of 18:3n-6 in Ot10-OE compared to Ot05-OE. Accordingly, the most reasonable assumption is that Ot10 has a preference for highly unsaturated \(\omega-3\) 16:4-Gal species.

Export of Plastidic PUFAs

Overexpression of each of the three Δ6-DES in O. tauri led to a similar and significant increase of 20:4n-6 in DGTA and TAGs and a specific and significant increase of 18:4n-3 in DGTA for Ot05-OE. We previously reported that the acyl-CoA pool was enriched in 18:3n-3 and 18:4n-3, whose amount varied according to the plastid C18-PUFA content (Degraeve-Guilbault et al., 2017). Together with the discovery of pΔ6-DES, these previous observations support that Δ6-DES products are exported from the plastid to the acyl-CoA pool in O. tauri. Though it cannot be excluded that pΔ6-DES have (limited) access to extraplastidic substrates, as suggested for the plastidic \(\omega-3\)-DES C. reinhardtii, it should be emphasized that the specificity of front-end DES is known to be more restricted than that of \(\omega-3\)-DES (Meessapyodsuk and Qiu, 2012; Nguyen et al., 2013; Wang et al., 2013).

Unexpectedly, pΔ6-DES-OE impacted extraplastidic GLs to a much greater extent in N. benthamiana. As mentioned above, these changes are likely arising from reallocation of xenobiotic plastidic Δ6-DES products to other membranes, possibly to circumvent deleterious effects, as well as from the low capacity of leaves to synthesize TAGs. A two-way exchange of lipids between the chloroplast and the extrachloroplastic compartments was proposed back in the mid-1980s to explain the altered 18:3 content of extraplastidic lipids observed in Arabidopsis plastidic \(\omega-3\)-DES (FAD7)-deficient mutants (Browse et al., 1986a). Most of the following work focused on the transfer of lipids and PUFAs from the ER to the chloroplast, establishing the idea that the reverse transport was negligible (Miquel and Browse, 1992; Li et al., 2016b). Nevertheless, the recent characterization of two Arabidopsis plastid lipase mutants highlighted that plastidic PUFAs indeed contributed to PUFa remodeling of extraplastidic GLs (Wang et al., 2017; Higashi et al., 2018).

Coregulation of DGDG and DGTA PUFAs Content

In O. tauri, 16:4 is exclusively at the sn-2 position in both plastidic and extraplastidic GLs, accounting for >70% and 20% of PDPT and DGTA species, respectively (Degraeve-Guilbault et al., 2017). Because 16:4-CoA is not detected in O. tauri, the origin of extraplastidic 16:4 remains puzzling. In this work, overexpression of Ot05 in O. tauri resulted in a pronounced decrease of 16:4 in DGDG, DGTA, and in TAGs. Several 16:4-species appeared related to these specific changes, including 18:3/16:4-DGTA, 20:4/16:4 DGTA, and sn-2-16:4 TAG species, the proportions of which appeared more reduced in Ot05 than the two other Δ6-OE lines. One possible, although speculative explanation for the synthesis of extraplastidic sn-2 16:4 lipids, would be that 18:3n-6/16:4-DGDG serves as precursor for 20:4n-6/16:4-DGTA; the reduction of 16:4-DGDG species would reverberate on sn-2-16:4-extraplastidic species including DGTA and TAGs. Galactosyl-Galactosyl-Galactolipid-transferase, not yet identified in microalgae, supports the production of DAG from DGDG in plants under freezing conditions (Moellering et al., 2010). Export of specific DGDG-species to extraplastidic membranes has been reported for plants and microalgae under phosphate starvation (Jouhet et al., 2004; Khozin-Goldberg and Cohen, 2006).

Significance of Plastidic Δ6-DES

The absence of alteration in growth and photosynthetic processes in O. tauri Δ6-DES-OE might be related
to certain compensatory mechanisms. These may include the increase of 16:0 and the decrease of 16:4, possibly alleviating any important changes in membrane unsaturation from the 18:3n-3 and 18:4n-3 variations that occur in Ot05-OE. On the other hand, photosynthesis defects were not detected in *Synechocystis* mutants devoid of 18:3n-3 and 18:4n-3, and such defects could only be unveiled in very specific conditions in the Arabidopsis mutant lacking trienoic PUFA (Gombos et al., 1992; Vijayan and Browne, 2002). There is, overall, very little evidence that plastidic PUFAs directly support photosynthetic processes (Mironov et al., 2012; Kugler et al., 2019).

The only plastidial front-end desaturase so far described was a Δ4-DES from *C. reinhardtii*, and its Cyt-b5 domain was shown to be active in vitro (Zäuner et al., 2012). Our data further show that a functional Cyt-b5 is a possible candidate is the Ferredoxin NADP oxidoreductase, is involved (Napier et al., 2003; Yang et al., 2015). It further indicates that another and desaturase domain in Cytb5 fused front-end DES (Napier et al., 2003). It further indicates that another redox partner, different from the eukaryotic Cyt-b5, is involved (Napier et al., 2003; Kumar et al., 2012; Meesapyodusk and Qiu, 2012). One possible candidate is the Ferredoxin NADPH-oxidoreductase (Yang et al., 2015).

The requirement of three Δ6-DES in a photosynthetic organism that displayed the most reduced set of genes points out the necessity of distinctly regulating the chloroplast and the cytosolic C18-PUFA pools. The existence of putative homologs of Δ6-DES in other microalgae species, such as haptophytes and dinoflagellates, might also be related to the co-occurrence of 18:4n-3 and 18:5n-3 in GaLs, and the prevalence of VLC-PUFA in extraplastidal lipids. For the diatom *Phaeodactylum tricornutum*, the putative Δ6-DES homolog might be involved in the desaturation of plastidic C16-PUFA, as was suspected long ago (Domergue et al., 2002). For most Mamiellophyceae, with the exception of *B. prasinus*, the two pΔ6-DES most likely arose from gene duplication. Ot10 would have evolved to restrict its specificity to a particular set of substrates. Transcriptional regulation of Ot05 by phosphate deprivation indicates that it is a physiological target under these conditions. The selectivity of Ot05 for SQDG, which is considered as a surrogate for PG under phosphate limitation, supports the use of Ot05 for conditions in which PG is metabolized to provide phosphorus. Ot10 and the putative α-3-DES transcriptional regulation might be targeted by other environmental cues, which still need to be uncovered.

**MATERIALS AND METHODS**

Chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

**Sequence Analyses**

DES domain-containing sequences were retrieved from NCBI genomic and transcriptomic data (Bioproject Accession: PRJNA304086 ID: 304086).

Annotated ORFs were manually checked for the completion of Nt sequences in species from the class Mamiellophyceae; cTPs were predicted with the tool PredAlgo (Tardif et al., 2012); alignment of Mamiellophyceae homologous sequences, identified by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search, was used to further determine putative cTPs assumed to correspond to the nonconserved Nt region (SnapGene trial version; Clustal Omega). These nonconserved regions were discarded for expression in *Saccharomyces cerevisiae* and *Synechocystis* sp. Codon-optimized sequences were obtained from Geneviz (Europe).

**Biological Material and Cultures**

*Ostrococcus tauri* (clonal isolate from OT05) was grown, as previously described (Degraeve-Guilbaud et al., 2017), in artificial sea-water, with either 5 or 35 μM of NaH2PO4, 0.5 mg mL⁻¹ of penicillin, and 0.25 mg mL⁻¹ of streptomycin. Centrifugation cycles (1,000 g, 5 min) were used to reduce bacterial contamination, and flow cytometry was then used to assess growth and bacterial contamination (Degraeve-Guilbaud et al., 2017). *Synechocystis* sp. PCC 6803 was grown according to the method described in Kojima et al. (2014). *Nitocira benthamiana* plants were cultivated in a greenhouse under controlled conditions (16-h:8-h photoperiod, 25°C). The *Agrobacterium tumefaciens* strain GV3101 was grown in Luria Broth medium at 30°C; the *S. cerevisiae* strain InvSc1 (MATa a::his3-11,15 leu2-3,112 tyr1-1 ura3-52; Invitrogen) was grown in synthetic dextrose medium (5 μl in a 50-mL flask, 30°C, 180 rpm; Erlenmeyer).

**Cloning Strategy**

PCR amplifications of DESORFs were achieved using QS Polymerase by two-step PCR on a cDNA matrix (primers are indicated in Supplemental Table S2). A Monarch DNA Gel Extraction kit was used as needed (New England Biolabs). Overexpression vectors were OtoLux for *O. tauri* (Moulager et al., 2010), pTHT2031S for *Synechocystis* sp. PCC 6803 (Kojima et al., 2014), GATEWAY destination vector pVT102-U-GW for *S. cerevisiae* (Domergue et al., 2010), and PK7W2C2D for *N. benthamiana* (Karimi et al., 2002). For subcellular localization, the final destination vector was pK7YW2G (Karimi et al., 2002) Nt-YFP-fusion was used. Subcloning was performed in pGEMT vector by restriction enzymes (Promega), in pUC57 for the codon-optimized sequence used in *Synechocystis* and for *S. cerevisiae* (GeneScript Biotech), and/or in pDONR 221 for GATEWAY cloning. Restriction cloning was used for cloning to *P. askani*, and ligase was used to introduce the synthetic gene with pTHT2031S In-Fusion HD Cloning Kit (Takara Bio). Sequencing was achieved by Geneviz.

Site-directed mutagenesis H → A of the HPGC motif of the Cyt-b5 domain was either performed with GeneScript (GeneScript Biotech; *N. benthamiana*) or using an In-Fusion HD Cloning Kit (Takara Bio) for *Synechocystis* after amplification using two mutagenic complementary primers for amplifying pTHT2031-O1H46A-S and pTHT2031-O1H20A-S from pTHT2031-O1X5-S and pTHT2031-O1H0-S, respectively. The mutated DNA sequence was validated for the correct modification using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies/Thermo Fisher Scientific).

**RNA and cDNA Preparation and Real-Time Quantitative PCR Analysis**

An RNeasy-Plus Mini Kit (Qiagen) was used for RNA purification. rDNA1 was used to remove contaminating DNA (DNA-Free Kit; Invitrogen), and cDNA was obtained using an iScript Reverse Transcription Supernmix kit (Bio-Rad). Real-time quantitative PCR reactions were performed in a CFX96 Real-Time System (Bio-Rad) using the GoTag qPCR Master mix (Promega; primers are indicated in Supplemental Table S3). The software CFX Manager (v.3.1; Bio-Rad) was used for data acquisition and analysis. The Ct method was used to normalize transcript abundance with the references mRNA EF1α (elongation factor), CAL (calmodulin), and Actin protein-related2. PCR efficiency ranged from 95% to 105%. A technical triplicate was used, and at least two independent experiments were performed.

**Genetic Transformation**

*O. tauri* electroporation was adapted from Correllou et al. (2009). Transgenics were obtained by electroporation and prescreened according to their luminescent level (Moulager et al., 2010). *S. cerevisiae* was transformed using a Plastidic Δ6-Desaturases in the Green Lineage.

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PEG/lithium acetate protocol, and FA supplementation was achieved. Control lines are transgenics of empty vectors. *N. benthamiana* leaves from 5-week-old plants were infiltrated with *A. tumefaciens* previously transformed by electroporation; p19 protein, to minimize plant post-transcriptional gene silencing, was used in all experiments. Briefly, *A. tumefaciens* transformants were selected with antibiotics (25 μg mL⁻¹ of gentamycin with 100 μg mL⁻¹ of spectinomycin or 50 μg mL⁻¹ of kanamycin). *A. tumefaciens* transformants were grown overnight, diluted to an OD₆₀₀ of 0.1 and grown to an OD₆₀₀ of 0.6 to 0.8. Cells were resuspended in 5 mL of sterilized water for a final OD of 0.4 and 0.2 for overexpression and subcellular localization experiments, respectively, and 1 mL was agroinfiltrated. Plants were analyzed 2 and 5 d after *A. tumefaciens* infiltration for subcellular localization experiments and for overexpression, respectively.

*Synechocystis* sp. PCC 6803 transformation was achieved by homologous recombination (Williams, 1988). Briefly, the plasmid was transformed into 10^9 concentrated cells of the ΔhelD strain collected at midlog phase. Subsequently, the cell was incubated at 30°C under white fluorescent lamps for 16 to 18 h and selected by 25 μg mL⁻¹ of chloramphenicol and 5 μg mL⁻¹ of spectinomycin on BG-11 solid media (1.5% [w/v] Bacto-agar; BD Diagnostics [VWR]).

**Lipid Analysis**

FA analyses for all organisms and further lipid analyses for *O. tauri* were achieved according to the method given in Degraeve-Guilbault et al. (2017). Organic solvents all contained butylhydroxytoluene as an antioxidant (0.001%). For *N. benthamiana*, frozen material (one leaf broken into pieces) was pre-incubated in hot isopropanol (3 mL, 75°C, 15 min, for Phospholipase D inhibition), and further extracted with 1 mL of CHCl₃ (using a T 25 digital Ultra-Turrax; IKA.com). Phase separation occurred upon addition of NaCl 2.5% (2,000×; 10 min). The pellet was re-extracted (3 mL of CHCl₃/CH₃OH 2:1 [v/v]); Organic phases were washed twice with 0.25 volume of CH₃OH/water (10^9 [v/v]). The lipid extract was evaporated under a nitrogen stream and resuspended in CHCl₃/CH₃OH 2:1, (v/v; 200 μL). Lipids were separated by high performance thin layer chromatography and chloroform/methanol/glacial acetic acid/water (85:12:12:1 [v/v/v/v]); For *Synechocystis* sp. (30 mg of dry weight and 3 mL of CHCl₃/CH₃OH 2:1 [v/v]), extraction was achieved using glass bead vortexing. High performance thin layer chromatography developments were achieved in the ADC2-chamber system (CAMAG) according to the method in Degraeve-Guilbault et al. (2017), except for *Synechocystis* sp. polar lipids (CHCl₃/CH₃OH/CH₃COOH/water 85:12:12:1 [v/v/v/v]; Sallal et al., 1990). Lipids were visualized and collected as previously described. MS analyses of *O. tauri* GL species was performed as in Abida et al. (2015). Purified lipids were introduced by direct infusion (electrospray ionization MS) into a trap-type mass spectrometer (LTQ-XL; Thermo Fisher Scientific) and identified by comparison with standards. Lipids were identified by tandem mass spectrometry analysis with their precursor ion or by neutral loss analyses. Positional analysis of FA in GLs was performed as in Degraeve-Guilbault et al. (2017).

**Confocal Microscopy**

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

**Photosynthesis Measurement**

Measurements were made using a PHYTO-PAM analyzer (Heinz Walz).

**Light Response of PSII Activity**

Rapid light-response curves (RLCs) of chlorophyll fluorescence of the cultures were achieved according to the method in Serôdio et al. (2006). Briefly, the cultures were exposed to 12 increasing actinic light levels (10-s light steps of 100 μE increase from 64 to 2064 μE), and the electron transport rates (ETRs) were calculated on each step to draw RLCs. The following parameters were extracted from the ETR-irradiance curve fitted to the experimental data: the initial slope of the curve (α), the light-saturation parameters (lₛ), and the maximum relative ETR.

**Photoinhibition Experiment**

Optimal conditions for PSII inhibition and recovery were adapted from Campbell and Tyystjärvi (2012). Cultures (50 mL, 3×) were maintained under fluorescent white light (low light: 30.4 ± 1.0 μE, white light) without agitation at 20.2 ± 0.2°C and moved to high light (117.6 ± 4.9 μE, blue LED) for 45 min (photoinhibition); photo recovery under initial condition was monitored for over 2 h. One-mL sampling was used to assess photosynthetic efficiency (quantum yield of photochemical energy conversion in PSI; Y corresponds to $Yield = df/Fm$).

**Accession Numbers**

These accession numbers have been deposited in Bioproject Accessions (https://www.ncbi.nlm.nih.gov/): *O. tauri* OTI95 PRJNA340486 (Transcriptome or Gene expression), *O. tauri* RCC 4221 PRJNA51609 (RefSeq Genome), *O. tauri* RCC115 PRJNA51609 (RefSeq Genome), Ostreococcus lucimarinus PRJNA19407 (RefSeq Genome), Bathycoccus prasinos PRJNA19407 (RefSeq Genome), Micromonas commoda PRJNA39769 (RefSeq Genome), and Micromonas pusilla PRJNA51515 (RefSeq Genome).

**Supplemental Data**

The following supplemental materials are available.

Supplemental Figure S1. Sequence features of *O. tauri* Δ6-DES candidates and of *Mamiellales* homologs.

Supplemental Figure S2. Alignment of *O*05 homologs in *Mamiellales* species.

Supplemental Figure S3. Alignment of *O*010 homologs in *Mamiellales* species.

Supplemental Figure S4. Alignment of Δ6-DES candidate and closest homologs from phylogenetically distant species.

Supplemental Figure S5. Heterologous expression in *S. cerevisiae*.

Supplemental Figure S6. Fatty-acyl profiles of *N. benthamiana* leaves transformed with *O. tauri* Δ6/Δ8-DES candidates.

Supplemental Figure S7. GL analyses of *N. benthamiana* leaves transformed with *O. tauri* Δ6-DES candidates.

Supplemental Figure S8. Selection of *O. tauri* Δ6-DES transgenics.

Supplemental Figure S9. FA-profile of best transgenics for each Δ6-DES.

Supplemental Figure S10. Growth of Δ6-DES overexpressing *O. tauri* lines.

Supplemental Figure S11. DGTA m/z species analysis of *O. tauri* Δ6-DES-OE.

Supplemental Figure S12. TAG m/z species analysis of *O. tauri* Δ6-DES-OE.

Supplemental Figure S13. TAG molecular species analysis.

Supplemental Figure S14. Photosynthetic activities of *O. tauri* Δ6-DES-OE.

Supplemental Table S1. *O. tauri* protein sequence features of FA desaturases.

Supplemental Table S2. Cloning primers.

Supplemental Table S3. Quantitative PCR primers table.

Supplemental Table S4. Photosynthetic parameters of *O. tauri* pΔ6-DES-OE.

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