ARTICLE TITLE

Functional screening of a novel Δ15 fatty acid desaturase from the coccolithophorid *Emiliania huxleyi*

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ABBREVIATIONS

Cyt b_5, cytochrome b_5; DGDG, digalactosyl diacylglycerol; ER, endoplasmic reticulum; FAME, fatty acid methyl ester; MGDG, monogalactosyl diacylglycerol; ORF, open reading flame; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; WT, wild type

ABSTRACT

The coccolithophorid *Emiliania huxleyi* is a bloom-forming marine phytoplankton thought to play a key role as a biological pump that transfers carbon from the surface to the bottom of the ocean, thus contributing to the global carbon cycle. This alga is also known to accumulate a variety of polyunsaturated fatty acids. At 25°C, *E. huxleyi* produces mainly 14:0, 18:4n-3, 18:5n-3 and 22:6n-3. When the cells were transferred from 25°C to 15°C, the amount of unsaturated fatty acids, i.e. 18:1n-9, 18:3n-3 and 18:5n-3, gradually increased. Among the predicted desaturase genes whose expression levels were up-regulated at low temperature, we identified a gene encoding a novel Δ15 fatty acid desaturase, EhDES15, involved in the production of n-3 polyunsaturated fatty acids in *E. huxleyi*. This desaturase contains a putative transit sequence for localization in chloroplasts and a Δ6 desaturase-like domain, but it does not contain a cytochrome b_5 domain nor typical His-boxes found in Δ15 desaturases. Heterologous expression of EhDES15 cDNA in cyanobacterium *Synechocystis* sp. PCC 6803 cells increased the level of n-3 fatty acid species, which are produced at low levels in wild-type cells grown at 30°C. The orthologous genes are only conserved in the genomes of prasinophytes and cryptophytes. The His-boxes conserved in orthologues varied from that of the canonical Δ15 desaturases. These results suggested the gene encodes a novel Δ15 desaturase responsible for the synthesis of 18:3n-3 from 18:2n-6 in *E. huxleyi*.

INTRODUCTION

Coccolithophorids (classified in division Haptophyta), a group of eukaryotic microalgae that produce coccolith, often form blooms in the early summer until mid-summer in the ocean, thought to contribute to the global carbon cycle [1,2,3]. They are believed to play an important role in the global climate, including warm temperature trends and ocean acidification, which is caused by an increase in CO_2 concentration in the atmosphere. A species of coccolithophore, *Emiliania huxleyi*, is used for numerous comparative physiological studies, since it is a contemporary species and grows well in laboratory cultures [4]. In accordance with lipid analyses, *E. huxleyi* accumulates very long chain ketones, namely alkenone [5] and polyunsaturated fatty acids (PUFAs), including all-*cis*Δ3,6,9,12,15-octadecapentaenoic acid (18:5n-3) [6]. Alkenones are methyl- or ethyl-ketones with C_{37}-C_{40} possessing 2-4 *trans*-type unsaturated bonds and are only detected in five genera from haptophytes [7]. The 18:5n-3 fatty acid has been identified in some species of haptophytes, dinoflagellates, raphidophytes and prasinophytes (division Chlorophyta); thus, it is used as a lipid biomarker for these species [7]. Among the haptophytes (besides *E. huxleyi*), *Isochrysis* sp. [8], *Chrysochromulina polylepis* [9], *Crystallolithus hyalinus* [10], *Hymenomonas elongate* [11], *Prymnesium saltans* and *Coccolithophora* sp. [12] produce 18:5n-3. However, the biological functions and synthetic pathway of 18:5n-3 remain unclear. One role of 18:5n-3 in the dinoflagellate, *Gymnodinium* cf. *mikimotoi*, is its toxic effect in the gills of its prey, sea bass *Dicenrarchus labrax* [13]. In *E. huxleyi*, 18:5n-3 is attached to glycolipids, such as mono- and di-galactosyl diacylglycerol (MGDG and DGDG) [6], indicative of thylakoid membrane localization and of a relationship with photosynthetic functions and machinery stability.

In contrast to fatty acid metabolism in haptophytes, C_{16} and C_{18} fatty acid species in higher plants and model microalgae including cyanobacteria have been well-studied. Genetic and biochemical analyses of mutants of the model microalgae *Chlamydomonas reinhardtii* have shown

that *de novo* synthesis of C_{16} and C_{18} fatty acid species are catalysed via the plastidial pathway: palmitic acid (16:0)-ACP and 18:0-ACP are produced via the plastidial fatty acid synthesis cycle, and 18:0-ACP is desaturated into oleic acid (18:1n-9)-ACP via a soluble Δ9 acyl-ACP desaturase. The 18:1n-9-ACP is then integrated into glycolipid species in the thylakoid membrane of the chloroplast, where it plays a role in MGDG, DGDG and sulfoquinovosyl diacylglycerol (SQDG) synthesis in combination with acyl-ACP transferase. Δ12 and Δ15 acyl-lipid desaturases are responsible for the synthesis of α-linolenic acid (18:3n-3) from 18:1n-9 [14,15]. In eukaryotic photosynthetic organisms, another pathway also exists to produce unsaturated C_{18} fatty acid species in the endoplasmic reticulum (ER) membrane, namely, the cytoplasmic pathway. Both the cytoplasmic pathway and the plastidial pathway are parallelly contributory in the organisms. For instance, *C. reinhardtii* possesses two set of Δ12 and Δ15 acyl-lipid desaturases of which are predicted to be localized in the ER membrane and in the chloroplast. These two sets of desaturases share structurally similar catalytic domains containing three conserved His-boxes, but they can be distinguished by their signal peptides for localization into different organelle and the electron donors. The desaturases located in the ER accept electrons for desaturation reaction from Cyt b_5 or intramolecular Cyt b_5 domain, whereas the chloroplast-localized desaturases utilize Fe_2-S_2 ferredoxin as an electron donor. Although the presence of the intramolecular Cyt b_5 domain is a characteristic feature in ER-localized desaturases, not all the ER-located desaturases possess the domain. In order to identify the localization of fatty acid desaturase without Cyt b_5 domain, it requires the further functional analyses, such as characterization by mutants' screening that affect fatty acid compositions [16,17,18] or investigating by heterologous functional expression in *e.g.* cells of *Saccharomyces cerevisiae*, which easily uptake a variety of fatty acids as substrates and incorporate them into their membrane lipids. Some desaturases in the haptophytes *E. huxleyi*, *Isochrysis galbana* and *Pavlova salina* were previously characterized by the heterologous

92 expression analyses in the yeast cells [19,20,21]. However, for the characterization of
93 chloroplast-located desaturase by heterologous expression, it is necessary to use a photosynthetic
94 organism as host cells because of requirement of the plastidial Fe_2-S_2 ferredoxin for supply of
95 electron.

96 In this study, we report the screening and functional characterization of a novel Δ15 acyl-lipid
97 desaturase from *E. huxleyi*, which is predicted to be localized in the chloroplast based on
98 heterologous expression in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). When we
99 expressed an *E. huxleyi* gene encoding a putative plastidial acyl-lipid desaturase homologous to Δ6
100 desaturase in *Synechocystis*, the cells of the transformants accumulated 18:3n-3 and 18:4n-3 and
101 showed decreased amounts of α-linoleic acid (18:2n-6) and γ-linolenic acid (18:3n-6). These results
102 suggested that the gene encodes a novel Δ15 desaturase responsible for the synthesis of 18:3n-3
103 from 18:2n-6 in *E. huxleyi*. In this report, we discuss PUFA metabolism in divergent microalgae and
104 the usability of cyanobacteria as a tool for functional analysis of plastidial desaturases.
105

MATERIALS AND METHODS

Organisms and culture conditions

Cells of the coccolithophorid *E. huxleyi* strain CCMP1516 were grown in 500 ml artificial seawater, Marine Art SF-1 (Tomita Seiyaku, Tokushima, Japan distributed by Osaka Yakken, Osaka, Japan), enriched with Erd-Schreiber's seawater containing 10 nM sodium selenite instead of soil extracts [22]. Cells were continuously illuminated by white fluorescent lamps (100 µmol photons m^{-2} s^{-1}) at 25°C with aeration. Growth of *E. huxleyi* was measured based on the OD at 750 nm or by counting cell numbers under microscopic observation (BX-50; Olympus, Tokyo, Japan).

For heterologous expression of the desaturase gene, a glucose-tolerant strain of the cyanobacterium *Synechocystis* was used as a host organism. Wild-type (WT) cells and all genetically transformed cells of *Synechocystis* were grown in BG11 media buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5) [23] at 30°C under continuous illumination by white fluorescent lamps at 70 µmol photons m^{-2} s^{-1} and aerated with 1% (v/v) CO_2-enriched air. All transformants were maintained in BG11 media solidified with 1.5% (w/v) Bacto-agar (Difco Laboratories, Detroit, MI) in the presence of 25 µg/ml kanamycin sulfate, 25 µg/ml spectinomycin dihydrochloride pentahydrate or 25 µg/ml chloramphenicol, depending on the selectable markers.

Lipid analysis

All glassware for lipid analyses were used after baking at 450°C for 3 hours to remove contamination. Cells of *E. huxleyi* and *Synechocystis* were collected by centrifugation (3,000 x *g* for 5 min) and stored at -80°C until lipid extraction. Total lipids were extracted with 5 ml methanol using ultrasonication in a water bath at 4°C. The homogenates were transferred to new glass test tubes with teflon-lined screw-caps. A total of 100 µl 500 µg/ml heptadecanoic acid (17:0) dissolved

130 in hexane was added to each sample as an internal standard. After complete drying using a
131 concentrating centrifuge (CC-105, Tomy Seiko, Tokyo, Japan), the pellet was re-suspended in 4 ml
132 0.1 M hydrochloric acid methanolic solution (Wako Pure Chemicals, Osaka, Japan). The tube was
133 tightly capped and heated at 100°C for 1 h in a water bath until free fatty-acids and acyl-groups in
134 lipids were saponified and methyl-esterified for conversion into fatty-acid methyl esters (FAMEs).
135 The resultant FAMEs were recovered with 4 ml *n*-hexane, and the acid-methanol phase was
136 re-extracted with 2 ml *n*-hexane and 2 ml deionized water. The hexane phases recovered were
137 evaporated, and the residues containing FAMEs were dissolved in 100 µl *n*-hexane.

138 To identify and quantify FAMEs, we applied 1 µL of the hexane solution to the
139 gas-chromatograph, GC-2014, equipped with a flame-ionization detector (Shimadzu, Kyoto, Japan).
140 Helium was used as a carrier at a constant flow rate of 1.25 ml/min in split-less mode. A column
141 CP-Sil5 CB (Agilent Technologies, Santa Clara, CA) was used at the following temperature; 60°C
142 for 1.5 min, an increase to 130°C at 20°C /min, a further increase to 300°C at 4°C/min, and holding
143 at 300°C for 25 min. FAMEs were identified based on retention time and confirmed using
144 commercial FAME standards (Nu-Chek Prep, Elysian, MN,). The amount of each FAME (pg/cell)
145 was measured by comparing the peak area of the total ion chromatogram with that of 17:0
146 methyl-ester as an internal standard and was normalized by cell number. To identify 18:3n-3 and
147 18:4n-3, which were newly synthesized by expression of the desaturase gene in *Synechocystis*, we
148 used a gas chromatograph, GC-2010, equipped with a mass spectrometer, QP-2010 (Shimadzu).
149 Analysis conditions were identical to those used for the FAMEs quantification, as described above.
150 We confirmed the retention times and mass spectrums between the standard methyl-esters of
151 18:3n-3 and 18:4n-3 (Sigma-Aldrich, Tokyo, Japan) and candidate methyl-esters of 18:3n-3 and
152 18:4n-3 observed in transformant cells.
153

RNA extraction

For the RNA extraction from *E. huxleyi*, we firstly cultured the cells at 25°C. When the OD_{750} reached at 0.2, the cells were transferred to 15°C and further cultured. At 24 and 48 h after transferring to 15ºC, cells were collected by centrifugation at 9,100 × *g* for 5 min at 4°C. Total RNAs were extracted using the Total RNA Isolation System (Promega, Madison, WI). mRNAs were isolated using the PolyATtract mRNA Isolation System (Promega). For the RNA extraction from *Synechocystis*, WT and transformants cells were inoculated into fresh BG11 medium at OD_{730} of 0.2 and the cultures were transferred into 22°C after growing for 16 h under standard growth conditions at 30°C. After 2 h culturing at 22°C and 30°C, the cultures were mixed with the same volume of ice-cold 10% (w/v) phenol-ethanol to prevent the degradation of RNAs, and cells were collected by centrifugation at 9,100 × *g* for 5 min at 4°C. The total RNAs were isolated using the TRIzol Max Bacterial RNA Isolation Kit (Invitrogen, Carlsbad, CA).

Plasmid construction and transformation

For heterologous expression of the desaturase gene, we constructed a plasmid for transformation of *Synechocystis* cells. pTCHT2031V [24; provided by Dr. Narikawa in the University of Tokyo], used to construct an expression plasmid, includes five DNA fragments in the following order: the upstream sequence of the *slr2031* gene, a chloramphenicol resistance gene cassette, the *trc* promoter sequence [25], the downstream sequence of the *slr2031* gene and the plasmid backbone from the pUC vector.

We isolated full-length cDNA for the fatty acid desaturase (Genbank Accession Number, EOD40666), which is thought to be involved in 18:5n-3 biosynthesis. mRNAs were isolated from *E. huxleyi* cells harvested 24 h after the cells were transferred from 25°C to 15°C as described above. A CapFishing Full-Length cDNA Premix Kit (Seegene, Seoul, Korea) was used to obtain full-length

cDNAs of the target gene. A gene specific primer (ATGCGCTTCAGGTGCTTGAC) designed based on the *E. huxleyi* genome sequence [26] was used for PCR amplification of the 5'-end of the transcript. The amplified DNA fragment was subcloned into the pGEM T-easy vector (Promega) and sequenced to determine the transcriptional start site of the desaturase gene. We synthesized an artificial gene sequence (Operon Biotechnologies, Tokyo, Japan) corresponding to the open reading frame of the desaturase gene lacking 74 amino acids of the N-terminus, which encompassed a putative signal and transit peptide, and optimized the codon usage to the host with additions of NdeI and HpaI recognition sites at the 5' and 3' termini, respectively. We amplified a DNA fragment of the spectinomycin resistance gene using the primers spr_F_BglII (GGAGATCTATCAATTCCCCTGCTCGCGC) and spr_R_BamHI (GGGGATCCTCCCAATTTGTGTAGGGCTT) and pAM1146 [27] as a template and subcloned the blunt-ended DNA fragment into the HpaI site at the 3' end of the synthetic gene. After excision of a DNA fragment containing the synthetic gene and the spectinomycin resistance gene cassette by NdeI and BamHI, we inserted the fragment into NdeI-BglII sites of pTCHT2031V to obtain the plasmid used for transformation of *Synechocystis*. The resulting plasmid was used to transform cells of WT and the *desB*-disruptant [28] *Synechocystis* by homologous recombination [29]. After verifying full segregation of the chromosome by PCR, fatty acid compositions and gene expression were analysed.

Quantification of mRNA

mRNAs from *E. huxleyi* and total RNAs from *Synechocystis* were reverse-transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time) (Takara Bio, Ohtsu, Japan) to obtain cDNAs. Semi-quantitative PCR was performed to measure EhDES15 mRNA levels in each 10 pg cDNA from *E. huxleyi* using the primers EhDES15_RT_F (GATGAAGCCCAACTTCATCTCCGTG) and

EhDES15_RT_R (ACTTGAGCTTTGCGGGGAGCGGGAA). As an internal control, a gene for Actin-related protein 3 (Actin3) was targeted using the same cDNA as template with the primers act_F (TACGAGGAGTATGGGCCTTC) and act_R (CTACATCGTGATTGCCGAGA).

Semi-quantitative PCR to measure the *desB* mRNA level was performed with each 10 pg cDNA samples from cells of WT and transformed *Synechocystis* as templates and the primers desB_RT_F (TCCAAGAGCTCAGAAACGCT) and desB_RT_R (GCTGAGATGACCAATCCAAT). Quantitative real-time PCR was also performed using the same cDNA samples with the primers desB_RT_F and desB_RT_R, SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio), and the PikoReal Real-Time PCR system (ThermoFisher Scientific, Waltham, MA). The results were normalized based on the expression level of the *rnpB* gene as an internal standard using primers rnpB_RT_F (GTAAGAGCGCACCAGCAGTATC) and rnpB_RT_R (TCAAGCGGTTCCACCAATC).

RESULTS

Fatty acid composition of *E. huxleyi*

We cultured *E. huxleyi* cells at 25°C. When the OD_{750} of the culture reached at 0.2, the culture was transferred to 15°C and further incubated. We withdrew aliquots of the cultures every 24 h for 6 days and measured the cell density (Fig. 1). Compared with cells maintained at 25°C, cell growth and fatty acid levels were not affected in cells grown at 15°C. The cell density at day 6 was 7.9 ± 0.6 × 10^6 (cells/ml) in the cultures grown at 15°C and 8.6 ± 1.8 × 10^6 (cells/ml) in the cultures grown at 25°C. We extracted total fatty acids from the cell aliquots and measured the fatty acid composition. Contents of total fatty acids at day 6 were 1.3 ± 0.2 and 1.5 ± 0.6 pg/cell in the cells grown at 15°C and 25°C, respectively (Supplemental Fig. 1). Under 25°C condition, 18:4n-3 content was slightly decreased depending on the growth stage, but other fatty acid contents were not changed during culturing (Fig. 2A). When the cultures were transferred to 15°C and incubated for 2 days, we observed an increase in 18:5n-3 (18.1 ± 3.2 mol%) and a decrease in 18:4n-3 (9.4 ± 0.8 mol%) contents (Fig. 2B). During further cultivation at low temperature, the content of 18:5n-3 gradually decreased to 12.9 ± 2.8 mol% by day 6 (similar to day 0). The 18:4n-3 content continuously decreased to 7.7 ± 0.3 mol% by day 6. Culturing for 6 days at 15°C dramatically increased 18:1n-9 and 18:3n-3 levels. Total content of C_{18} species at day 0 was about 44 mol%. This value was decreased to 39.0 ± 2.8 mol% at day 6 in 25°C and increased to 48.7 ± 1.1 mol% at day 6 in 15°C cultured condition. Cells also contained high amounts of 14:0 and 22:6*n*-3 fatty acids: 21.9 ± 1.1 and 21.3 ± 2.4 mol%, respectively, at day 0 (Supplemental Fig. 2). The saturated fatty acid 14:0 and 16:0 gradually decreased under low temperatures. A decrease in saturated fatty acids and an increase in mono-unsaturated fatty acids under low temperature condition have been observed in several plants and microalgae [30]. Although the 22:6n-3 fatty acid, which was highly accumulated at 25°C, was not affected by the temperature shift, another long-chain saturated fatty

238 acid, 22:0 was increased. These changes in fatty acid compositions under lower temperature
239 conditions were not observed in cultures maintained at 25°C (except 14:0 levels). Both the content
240 and absolute amount of 14:0 gradually increased at 25°C (Supplemental Fig. 2A and 3A). The
241 decrease in 14:0 and 16:0 contents observed at 15°C may be due to the increase in 18:1n-9 and
242 18:5n-3 contents. A compositional change in fatty acids stimulated under the low temperature was
243 observed for C_{18} fatty acid species: 18:1n-9, 18:3n-6, 18:4n-3 and 18:5n-3 in *E. huxleyi*. According
244 to the fractionation and quantification of each lipid class extracted from *E. huxleyi*, MGDG and
245 phosphatidylcholine (PC) were two major lipid classes in total lipids in *E. huxleyi* [6]. MGDG was
246 dominated by the acyl-chains of C_{18} fatty acid species containing 18:1, 18:3n-3, 18:4n-3 and
247 18:5n-3 at 70.7% among all fatty acids esterified to the glycerol backbone. PC was also an abundant
248 lipid class and contained only 18:1 at 2.4% of all acyl chains in PC. Thus, synthesis and
249 desaturation of the C_{18} PUFAs may have occurred in the chloroplasts. We then investigated
250 desaturases involved in the synthesis of C_{18} PUFAs in the chloroplasts.
251
252 **Genomic search for desaturase genes**
253 The whole genome sequence of *E. huxleyi* strain CCMP 1516 has been reported previously [26].
254 According to the KOG (eukaryotic orthologous groups) annotation by JGI, there are 29 genes
255 encoding proteins similar to the desaturase domain. Eighteen of these genes did not possess the
256 putative Cyt b_5 domain. We then classified the 18 proteins into four groups, Δ9, Δ12, Δ15 and Δ6,
257 based on similarities to the known plastid-type desaturases from cyanobacteria (Supplemental Table
258 1). Although Read and co-workers [26] annotated a Δ15 desaturase (EOD29061), two Δ12
259 desaturases (EOD07051, EOD26922) and a Δ6 desaturase (EOD40828) based on sequence
260 similarities, while 14 proteins remained unannotated. We further examined the protein, EOD40666,
261 hereafter referred to as EhDES15, which contains a homologous region to the cyanobacterial Δ6

desaturase. Interestingly, a BLAST search using the amino acid sequence of EhDES15 identified orthologous proteins in prasinophytes genomes (Fig. 3), such as *Ostreococcus lucimarinus* [31], *Micromonas pusilla* [32] and *Bathycoccus prasinos* [33]. *O. lucimarinus* and *M. pusilla* are also known to produce 18:5n-3 [34,35]. Thus, EhDES15 may play a role in 18:5n-3 production. EhDES15 is a protein of 448 amino acids showing partial similarity to the Δ6-fatty acid desaturase. Multiple alignment of amino acid sequences of EhDES15 and the orthologues was drawn (Fig. 3A). Although EhDES15 contains a sequence section similar to a posterior half of the Δ6 desaturase-like protein domain, whole amino acid sequences of EhDES15 and its orthologous proteins are well conserved. Particularly, regions including three conserved His-boxes were well conserved. Additionally, EhDES15 and the orthologues possess N-terminal extensions with very low similarities and various lengths. Haptophyte is a secondary plant which may acquire plastid via secondary endosymbiosis. As a consequence of evolutionary development, its chloroplast is surrounded by four envelopes. And the outermost envelope membrane is composed of the ER. Therefore, the nuclear-encoded plastid-targeting proteins contain bipartite sequences consisting of signal peptide for passing through ER membrane followed by a transit peptide for incorporation into the plastid [36]. For the prediction of these bipartite signal and transit peptides, firstly SignalP [37] was used to estimate the signal peptide and ChloroP [38] were used to estimate the transit peptide after elimination of the predicted signal peptide. SignalP deduced amino acid residues 1 to 27 of EhDES15 as a signal peptide. But according to an example from another secondary alga, diatom *Phaeodactylum tricornutum*, "ASA-FAP" is the probable cleavage site (F at the +1 position is cleaved) and the phenylalanine residue can be substituted by leucine and "AP" is exchangeable [39]. Therefore, we estimated the 1-25 residues (ASA-L) as a signal peptide for plastid localization of EhDES15. ChloroP deduced amino acid residues 26-34 as a putative transit peptide for plastid transportation (Fig. 3A). Because this is relatively short, we estimated the mature protein started from 75 residues

286 according to the similarity to orthologues. Phylogenic tree of EhDES15 and its orthologues and Δ12,
287 Δ15 and Δ6 desaturase families also showed a sister relationship between EhDES15 and Δ6
288 desaturase family (Fig. 3B).

289 Since the decrease in culture temperature enhanced the production of 18:1n-9, 18:3n-3 and
290 18:5n-3 in *E. huxleyi* (Fig. 2), accumulation of mRNAs of the desaturases involved in desaturation
291 of these fatty acids would also likely be upregulated. Semi-quantitative PCR clearly showed
292 up-regulation of EhDES15 mRNA at 15°C (Fig. 4). Transcriptomic analysis, sequencing and
293 comparison of total cDNA reverse-transcribed from mRNA extracted from 25°C- and
294 15°C-cultured *E. huxleyi* cells also supported these results. mRNA levels were induced
295 approximately 3.5-fold compared with those from the 25°C conditions (Fig. 4, Araie et al.,
296 unpublished data). These results indicated that EhDES15 contributed to PUFA production induced
297 under low temperature conditions in *E. huxleyi*. We then performed a functional characterization of
298 EhDES15 using a heterologous expression system.
299
300 **Heterologous expression of EhDES15 in *Synechocystis* cells**
301 Since EhDES15 possesses a predicted transit-peptide for transportation into plastid and does not
302 contain Cyt b_5 domain, Fe_2-S_2 ferredoxin is likely required as an electron donor for the desaturation
303 reaction. Thus, we used *Synechocystis* cells as a host for heterologous expression. *Synechocystis* is a
304 unicellular photosynthetic microorganism used as a model for photosynthesis studies, since it is
305 highly competent for homologous recombination. This organism contains four desaturases, Δ9, Δ12,
306 Δ6 and Δ15, and produces 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3 and 18:4n-3 [40].
307 A codon-optimized EhDES15 gene lacking the 74 amino acid-peptide serving as putative
308 bipartite signal and transit peptides at the N-terminus was synthesized and expressed heterologously
309 in *Synechocystis* cells under control of the *trc* promoter [25]. At 30°C, the fatty acid composition

was investigated in cells expressing EhDES15 gene and WT cells. The amounts of 18:2n-6 and 18:3n-6 significantly decreased, and those of 18:3n-3 and 18:4n-3 increased in cells expressing EhDES15 gene (Fig. 5A and B). The 18:3n-3 and 18:4n-3 fatty acids were predominantly synthesized in WT cells cultured at low temperatures; however, they were present at low levels in cells cultured at 30°C, which we used in this study [41] (Fig. 5C and D). The two peaks on the chromatogram corresponding to methyl-esters of 18:3n-3 and 18:4n-3 were confirmed by a gas chromatograph equipped with a mass spectrometry with standard compounds (data not shown).

The genome of *Synechocystis* originally possesses a gene for Δ15 desaturase (*desB*, *sll1441*). Expression of the *desB* gene has been well-characterized and is induced in *Synechocystis* cells grown below 26°C [28]. We examined levels of *desB* mRNA in WT and transformant cells grown at 30°C or at 22°C for 2 h after culturing in 30°C by semi-quantitative PCR and quantitative real-time PCR analysis (Fig. 5E and F). Under 30°C condition, the *desB* mRNA was not detected by semi-quantitative PCR but detected by quantitative real-time PCR. By 2 h exposure of cold shock, the *desB* expression was induced both in WT and transformed cells, and expression levels in both cells were almost identical. These results indicated that the accumulation of PUFAs possessing an unsaturated bond at the Δ15 position in transformant cells at 30°C was due to EhDES15 activity, but not due to the unexpected expression of the native *desB* gene after transformation of the EhDES15 gene. These results were confirmed based on complementation analysis. We constructed the EhDES15-expressing *Synechocystis* mutant lacking the *desB* gene. The transformant cells accumulated 18:3n-3 and 18:4n-3 (Fig. 6D and E).

DISCUSSION

Fatty acid composition of *E. huxleyi*

The unicellular haptophyta, *E. huxleyi*, synthesizes many species of polyunsaturated fatty acids including 18:3n-3, 18:4n-3, 18:5n-3, 20:5n-3 and 22:6n-3. Excluding 18:5n-3, these fatty acid species are widely distributed in microalgae [12]. The 18:5n-3 fatty acid is specifically observed in secondary plants of the red lineage, such as haptophytes, dinophytes and raphidophytes, and in only one group of the green microalgae, prasinophytes [7,34,35]. We first observed changes in PUFA composition at low temperature in *E. huxleyi* (Fig. 2B). Fatty acid profiles indicated that low temperature stimulated expression of genes for desaturases, which catalysed the biosynthesis of 18:3n-3 and 18:5n-3, because the concentrations of these PUFAs increased after temperature decrease from 25°C to 15°C. The 18:3n-3 fatty acid gradually increased from day 1 to 3 after the temperature decreased from 25°C to 15°C. From days 4 to 6, the amount of 18:3n-3 did not change significantly, suggesting that expression of the Δ15 desaturase was transiently induced at low temperatures. The amount of 18:5n-3 increased transiently after cold shock and gradually decreased after 2 days (Fig. 2B). These two PUFAs and a mono-unsaturated fatty acid, 18:1n-9, may be involved in the regulation of membrane fluidity for low temperature acclimation in *E. huxleyi*.

Structure of the novel Δ15 desaturase in *E. huxleyi*

In higher plants and microalgae, Δ15 (ω3) desaturase catalyses the synthesis of 18:3n-3 and 18:4n-3 from 18:2n-6 and 18:3n-6, respectively, and is thereby involved in synthesis of the highly PUFAs, such as penta- or hexaenoic acids. In this study, we characterized a novel plastidial Δ15 desaturase from the *E. huxleyi* genome. Although the actual localization of the protein remains unknown, it is predicted to be in the thylakoid membrane or the chloroplast envelope, since EhDES15 possesses a putative transit sequence and was functional in *Synechocystis*, which contains only the

ferredoxin-type acyl-lipid desaturase [40].

We also found that the Δ15 desaturase was conserved in the genomes of prasinophytes, *Ostreococcus*, *Micromonas* and *Bathycoccus*, and in a cryptophyta, *Guillardia theta*. In contrast to haptophytes as a secondary plant derived from the red lineage, prasinophytes are primary plants known as a primitive group of the Cholorphyta lineage. According to the lipid profiles of *Ostreococcus* and *Micromonas*, both synthesize 18:3n-3, similar to *E. huxleyi* [34,35]. It is indicated that orthologues of EhDES15 in *Ostreococcus* and *Micromonas* catalyse the same Δ15 desaturation reaction to produce 18:3n-3 *in vivo*. Furthermore, both prasinophytes are known to be 18:5n-3 producers. We hypothesized that the key enzyme responsible for the synthesis of 18:5n-3 catalysing the desaturation of 18:4n-3 should be conserved among these algae. Ahmann and co-workers [34] identified ER-type Δ4 desaturase which introduces an unsaturated bond at the Δ4 position of 22:5n-3 to produce 22:6n-3 in *Ostreococcus*. Interestingly, this enzyme could also introduce an unsaturated bond at the Δ3 position of 18:4n-3 to produce 18:5n-3 when the gene was expressed in yeast cells under the supplementation of 18:4n-3 as substrate. Although the lipid class that contains 18:5n-3 remains unknown in *Ostreococcus* cells, it may synthesize 18:5n-3 in the ER membrane based on the wide substrate specificity of the putative Δ4/Δ3 desaturase. Because a stable transformation method has been established in *Ostreococcus* [42], it is possible to identify the desaturase involved in 18:5n-3 biosynthesis.

A phylogenic tree of EhDES15 and corresponding enzymes was constructed with well-characterized Δ15 and Δ6 fatty-acid desaturases in higher plants and microalgae (see Fig. 3B). The tree showed that EhDES15 and orthologues can be classified into a monophyletic group and are separated far from the canonical group including the Δ15 desaturase, as reported previously. Because of their homologies to the Δ6 fatty acid desaturase domain, the position was thought to be

more closely related to the Δ6 than the Δ15 desaturases. However, the length of the branch was long between EhDES15 and Δ6 desaturases. The difference between the novel Δ15 family and the typical Δ15 and Δ6 desaturases was also observed in the amino acid residues in the three His boxes. The amino acid sequence of EhDES15 contains three predicted His-boxes (HHTCH, HNHLHH and YQIEHH), which were well-conserved in orthologues from prasinophytes, and these sequences varied greatly from the cyanobacterial Δ6 (HDXNH, HXXXHH and QXXXHH) and Δ15 (HDCGH, HXXXXXHRTHH and HHXXXXHVAHH) [43]. These results indicate that EhDES15 may be a novel type of Δ15 desaturase.

Synechocystis as a tool for desaturase characterization

Although screening and characterization of ER-located desaturases for EPA or DHA biosynthesis in microalgae, are often performed using heterologous expression systems in yeast cells [19,20,21], few studies have been done on plastid-located fatty acid desaturases using heterologous expression in the host cells of the photosynthetic organism. ω6 desaturase from a higher plant, *Brassica napus*, was identified based on expression in a cyanobacterium, *Synechococcus* [44]. Domergue and colleagues also used the cells of *Synechococcus* to characterize the substrate specificity of Δ12 plastidial desaturase from a diatom, *Phaeoductylum triconutum* [45], because *Synechococcus* only produces 18:1n-9 as an unsaturated fatty acid. In this study, we used another cyanobacterium, *Synechocystis*, to provide ferredoxin and characterize the plastid type fatty acid desaturase. *Synechocystis* can synthesize several C_{18} unsaturated fatty acids, including 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3 and 18:4n-3, depending on the growth temperature [41], and all acyl-lipid desaturases have been characterized [40]. The plastidial Δ15 (ω3) desaturase from the sunflower was identified by the gene expression in the model cyanobacterium, *Synechocystis* [46]. Therefore,

401 using mutant lines lacking each desaturase gene and by controlling the culture temperature, we can
402 obtain any C_{18} substrate *in vivo* to measure substrate specificity of the plastidial desaturase, which
403 can facilitate functional analysis of numerous and divergent plastidial desaturases from non-model
404 photosynthetic organisms. Δ9, Δ12 and Δ6 desaturases in *E. huxleyi* have not been identified, and
405 many desaturase genes have not been identified in the microalgal genome. Heterologous expression
406 of genes in cyanobacteria may allow us to identify and characterize these unknown desaturases.
407

REFERENCES

[1] J.C. Orr, V.J. Fabry, O. Aumont, L. Bopp, S.C. Doney, R.A. Feely, et al., Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms, Nature 437 (2005) 681–686.

[2] S. Fukuda, I. Suzuki, T. Hama, Y. Shiraiwa, Compensatory response of the unicellular-calcifying alga *Emiliania huxleyi* (Coccolithophoridales, Haptophyta) to ocean acidification, J. Oceanogr. 67 (2011) 17–25.

[3] A. Winter, R. Jordan, P. Roth, Biogeography of living coccolithophores in ocean waters, in: A. Winter, W.G. Siesser (Eds.) Coccolithophores, Cambridge University Press, Cambridge, UK, 1994: pp. 161–177.

[4] E. Paasche, A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions, Phycologia 40 (2001) 503–529.

[5] J.K. Volkman, D.A. Everitt, D.I. Allen, Some analyses of lipid classes in marine organisms, sediments and seawater using thin-layer chromatography—flame ionisation detection, J. Chromatogr. A. 356 (1986) 147–162.

[6] M.V. Bell, D. Pond, Lipid composition during growth of motile and coccolith forms of *Emiliania huxleyi*, Phytochemistry 41 (1996) 465–471.

[7] J.K. Volkman, S.M. Barrett, S.I. Blackburn, M.P. Mansour, E.L. Sikes, F. Gelin, Microalgal biomarkers: A review of recent research developments, Org. Geochem. 29 (1998) 1163–1179.

[8] S.M. Renaud, H.C. Zhou, D.L. Parry, L.-V. Thinh, K.C. Woo, Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., *Nitzschia closterium*, *Nitzschia paleacea*, and commercial species *Isochrysis* sp. (clone T.ISO), J. Appl. Phycol. 7 (1995) 595–602.

[9] U. John, U. Tillmann, L. Medlin, A comparative approach to study inhibition of grazing and lipid composition of a toxic and non-toxic clone of *Chrysochromulina polylepis* (Prymnesiophyceae), Harmful Algae 1 (2002) 45–57.

[10] J.K. Volkman, D.J. Smith, G. Eglinton, T.E.V. Forsberg, E.D.S. Corner, Sterol and fatty acid composition of four marine haptophycean algae, J. Mar. Biol. Assoc. U. K. 61 (1981) 509–527.

[11] A.-C. Viso, J.-C. Marty, Fatty acids from 28 marine microalgae, Phytochemistry. 34 (1993) 1521–1533.

[12] I. Lang, L. Hodac, T. Friedl, I. Feussner, Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection, BMC Plant Biol. 11 (2011) 124.

[13] F. Sola, A. Masoni, B. Fossat, J. Porthé-Nibelle, P. Gentien, G. Bodennec, Toxicity of fatty acid 18:5n3 from *Gymnodinium* cf. *mikimotoi*: I. morphological and biochemical aspects on *Dicentrarchus labrax* gills and intestine, J. Appl. Toxicol. 19 (1999) 279–284.

[14] N. Sato, S. Fujiwara, A. Kawaguchi, M. Tsuzuki, Cloning of a gene for chloroplast ω6 desaturase of a green alga, *Chlamydomonas reinhardtii*, J. Biochem. 122 (1997) 1224–1232.

[15] H.M. Nguyen, S. Cuiné, A. Beyly-Adriano, B. Légeret, E. Billon, P. Auroy, et al., The green microalga *Chlamydomonas reinhardtii* has a single ω-3 fatty acid desaturase which localizes to the chloroplast and impacts both plastidic and extraplastidic membrane lipids, Plant Physiol. 163 (2013) 914-928.

[16] J. Browse, L. Kunst, S. Anderson, S. Hugly, C. Somerville, A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase, Plant Physiol. 90 (1989) 522–529.

[17] L. Kunst, J. Browse, C. Somerville, A mutant of *Arabidopsis* deficient in desaturation of palmitic acid in leaf lipids, Plant Physiol. 90 (1989) 943–947.

[18] M. Miquel, J. Browse, *Arabidopsis* mutants deficient in polyunsaturated fatty acid synthesis

Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase, J. Biol. Chem. 267 (1992) 1502–1509.

[19] O. Sayanova, R.P. Haslam, M.V. Calerón, N.R. López, C. Worthy, P. Rooks, et al., Identification and functional characterisation of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore *Emiliania huxleyi*, Phytochemistry 72 (2011) 594–600.

[20] S.L. Pereira, A.E. Leonard, Y.-S. Huang, L.-T. Chuang, P. Mukerji, Identification of two novel microalgal enzymes involved in the conversion of the ω3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid, Biochem. J. 384 (2004) 357–366.

[21] T. Shi, A. Yu, M. Li, X. Ou, L. Xing, M. Li, Identification of a novel C22-Δ4-producing docosahexaenoic acid (DHA) specific polyunsaturated fatty acid desaturase gene from *Isochrysis galbana* and its expression in *Saccharomyces cerevisiae*, Biotechnol. Lett. 34 (2012) 2265–2274.

[22] A. Danbara, Y. Shiraiwa, The requirement of selenium for the growth of marine coccolithophorids, *Emiliania huxleyi*, *Gephyrocapsa oceanica* and *Helladosphaera* sp. (Prymnesiophyceae), Plant Cell Physiol. 40 (1999) 762–766.

[23] R. Y. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and properties of unicellular blue-green algae (order Chroococcales), Bacteriol Rev. 35 (1971) 171–205.

[24] T. Ishizuka, T. Shimada, K. Okajima, S. Yoshihara, Y. Ochiai, M. Katayama, et al., Characterization of cyanobacteriochrome TePixJ from a thermophilic cyanobacterium *Thermosynechococcus elongatus* strain BP-1, Plant Cell Physiol. 47 (2006) 1251–1261.

[25] E. Amann, J. Brosius, 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*, Gene 40 (1985) 183–190.

[26] B.A. Read, J. Kegel, M.J. Klute, A. Kuo, S.C. Lefebvre, F. Maumus, et al., Pan genome of the phytoplankton *Emiliania* underpins its global distribution, Nature 499 (2013) 209–213.

480 [27] N.F. Tsinoremas, A.K. Kutach, C.A. Strayer, S.S. Golden, Efficient gene transfer in
481 *Synechococcus* sp strains PCC 7942 and PCC 6301 by interspecies conjugation and chromosomal
482 recombination, J Bacteriol. 176 (1994) 6764–6768.
483 [28] T. Sakamoto, D.A. Los, S. Higashi, H. Wada, I. Nishida, M. Ohmori, N. Murata, Cloning of ω3
484 desaturase from cyanobacteria and its use in altering the degree of membrane-lipid unsaturation,
485 Plant Mol Biol. 26 (1994) 249–263.
486 [29] J.G.K. Williams, Construction of specific mutations in photosystem II photosynthetic reaction
487 center by genetic engineering methods in *Synechocystis* 6803, Meth. Enzymol. 167 (1988)
488 766–778.
489 [30] I. Nishida, N. Murata, Chilling sensitivity in plants and cyanobacteria: The crucial contribution
490 of membrane lipids, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 541–568.
491 [31] E. Derelle, C. Ferraz, S. Rombauts, P. Rouzé, A.Z. Worden, S. Robbens, et al., Genome
492 analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features,
493 Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11647–11652.
494 [32] A.Z. Worden, J.-H. Lee, T. Mock, P. Rouzé, M.P. Simmons, A.L. Aerts, et al., Green evolution
495 and dynamic adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*, Science
496 324 (2009) 268–272.
497 [33] H. Moreau, B. Verhelst, A. Couloux, E. Derelle, S. Rombauts, N. Grimsley, et al., Gene
498 functionalities and genome structure in *Bathycoccus prasinos* reflect cellular specializations at the
499 base of the green lineage, Genome Biol. 13 (2012) R74.
500 [34] K. Ahmann, M. Heilmann, I. Feussner, Identification of a Δ4-desaturase from the microalga
501 *Ostreococcus lucimarinus*, Eur. J. Lipid Sci. Technol.. 113 (2011) 832–840.
502 [35] G.A. Dunstan, J.K. Volkman, S.W. Jeffrey, S.M. Barrett, Biochemical composition of
503 microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 2. Lipid classes and

504 fatty acids, J. Exp. Mar. Biol. Ecol. 161 (1992) 115–134.
505 [36] K. Ishida, Protein targeting into plastids: a key to understanding the symbiogenetic acquisitions
506 of plastids, J. Plant Res. 118 (2005) 237–245.
507 [37] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, SignalP 4.0: discriminating signal
508 peptides from transmembrane regions, Nat. Meth. 8 (2011) 785–786.
509 [38] O. Emanuelsson, H. Nielsen, G.V. Heijne, ChloroP, a neural network-based method for
510 predicting chloroplast transit peptides and their cleavage sites, Protein Sci. 8 (1999) 978–984.
511 [39] A. Gruber, S. Vugrinec, F. Hempel, S.B. Gould, U.-G. Maier, P.G. Kroth, Protein targeting into
512 complex diatom plastids: functional characterisation of a specific targeting motif, Plant Mol. Biol.
513 64 (2007) 519–530.
514 [40] D.A. Los, N. Murata, Membrane fluidity and its roles in the perception of environmental
515 signals, Biochim. Biophy. Acta 1666 (2004) 142–157.
516 [41] H. Wada, N. Murata, Temperature-induced changes in the fatty acid composition
517 of the cyanobacterium, *Synechocystis* PCC6803, Plant Physiol. 92 (1990) 1062–1069.
518 [42] G. van Ooijen, K. Knox, K. Kis, F.-Y. Bouget, A.J. Millar, Genomic transformation of the
519 picoeukaryote *Ostreococcus tauri*, J. Vis. Exp. 65 (2012) e4074.
520 [43] D.A. Los, N. Murata, Structure and expression of fatty acid desaturases, Biochim. Biophys.
521 Acta 1394 (1998) 3–15.
522 [44] W.D. Hitz, T.J. Carlson, J.R.B. Jr, A.J. Kinney, K.L. Stecca, N.S. Yadav, Cloning of a
523 higher-plant plastid ω-6 fatty acid desaturase cDNA and its expression in a cyanobacterium, Plant
524 Physiol. 105 (1994) 635–641.
525 [45] F. Domergue, P. Spiekermann, J. Lerchl, C. Beckmann, O. Kilian, P.G. Kroth, et al., New
526 insight into *Phaeodactylum tricornutum* fatty acid metabolism. cloning and functional
527 characterization of plastidial and microsomal Δ12-fatty acid desaturases, Plant Physiol. 131 (2003)

528 1648–1660.
529 [46] M. Venegas-Calerón, A.M. Muro-Pastor, R. Garcés, E. Martínez-Force, Functional
530 characterization of a plastidial omega-3 desaturase from sunflower (*Helianthus Annuus*) in
531 cyanobacteria, Plant Physiol. Biochem. 44 (2006) 517‑525.
532 [47] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of
533 progressive multiple sequence alignment through sequence weighting, position-specific gap
534 penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673 –4680.
535 [48] K. Tamura, S. Glen, P. Daniel, F. Alan, K. Sudhir. MEGA6: molecular evolutionary genetics
536 analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725-2729.
537

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544 **FIGURE LEGENDS**

545

546 **Fig. 1. Growth of *E. huxleyi* cells under 25°C and 15°C conditions**

547 Closed and open circles indicate cell densities at 25°C and 15°C, respectively. Each point represents
548 the average for three experiments, and the error bars represent the means ± SD.

549

550 **Fig. 2. C_{18} fatty acid composition of *E. huxleyi***

551 Changes in C_{18} fatty-acid levels in cells cultured continuously at 25°C (A) or cells transferred from
552 25°C to 15°C at day 0 (B). Each bar represents the average for triplicate experiments, and the error
553 bars represent the means ± SD. The values for all fatty acids are shown in Supplemental Table 2 and
554 3, and Supplemental Fig. 2 and 3.

555

556 **Fig. 3. Phylogenic analysis of desaturases of microalgae and plants**

557 Multiple alignment of EhDES15 and orthologous proteins in microalgae (A). Ehux, *E. huxleyi*
558 CCMP 1516 EhDES15 (EOD40666); Oluc, *Ostreococcus lucimarinus* (ABO95258); Bpra,
559 *Bathycoccus prasinos* predicted protein (CCO66881); Mpus, *Micromonas pusilla* CCMP1545
560 (EEH60489); Gthe, *Guillardia theta* CCMP2712 (EKX48065). The predicted signal peptide (SP)
561 and transit peptide (TP) in EhDES15 are enclosed by black and grey rectangles, respectively. Boxes
562 indicate tentative His-boxes, and asterisks indicate histidine residues in the boxes. The region with
563 homology to the Δ6 fatty acid desaturase-like domain is shown by dashed upperlines. The
564 alignment was drawn by the ClustalW program with full-length amino acid sequences [47].
565 Unrooted phylogenic tree of the desaturase family was drawn by MEGA 6 software [48] (B). Ehux,
566 Gthe, Mpus, Oluc, Bpra: refer to the legend of Fig. 3A. Ss6, Ss12 and Ss15 represent *Synechocystis*
567 Δ6 (BAK50679), Δ12 (BAK50342) and Δ15 desaturase (BAK50475), respectively. Pt6, Pt12_1,

568 Pt12_2 represent *Phaeodactylum tricornutum* Δ6 (AAL92563) and two Δ12 desaturases
569 (AAO23565, AAO23564), respectively. Tp6, Tp12_1, Tp12_2: *Thalassiosira pseudonana* Δ6
570 (AAX14505) and two Δ12 desaturases (EED90922, EED93612); Aa6: *Aureococcus*
571 *anophagefferens* Δ6 desaturase (EGB07085); Pp6: *Physcomitrella patens* Δ6 desaturase
572 (CAA11032); At12p, At12e, At15p, At15e: *Arabidopsis thaliana* plastid-localized Δ12 (AAL24186),
573 ER-localized Δ12 (AAA32782), plastid-localized Δ15 (AEE75009), ER-localized Δ15 desaturase
574 (AEC08330), respectively; Cr12p, Cr12e, Cr15p: *Chlamydomonas reinhardtii* plastid-localized Δ12
575 (BAA23881), ER-localized Δ12 (EDP04777), and plastid-localized Δ15 desaturase (EDP09401),
576 respectively; Cv12e, Cv15e: *Chlorella vulgaris* ER-localized Δ12 (BAB78716), Δ15 desaturase
577 (BAB78717); Bj15p: *Brassica juncea* plastid-localized Δ15 desaturase (CAB85467); Nt15p:
578 *Nicotiana tabacum* plastid-localized Δ15 desaturase (BAC01274); Pa15p: *Picea abies*
579 plastid-localized Δ15 desaturase (CAC18722); *Triticum aestivum* ER-localized Δ15 desaturase
580 (BAA28358); Os15e: *Oryza sativa* ER-localized Δ15 desaturase (BAA11397); Mp6: *Marchantia*
581 *polymorpha* Δ6 desaturase (AAT85663); Ot6: *Ostreococcus tauri* Δ6 desaturase (AAW70159).
582
583 **Fig. 4. Accumulation of the EhDES15 gene transcript at low temperature.**
584 The relative ratio was calculated based on RPKM (read per kilobase per million mapped reads)
585 values between 15°C and 25°C according to transcriptomic analysis (Araie et al., unpublished data).
586 RPKM value represents the detection frequency in the transcriptome of a certain gene normalized
587 by its ORF length. The cells were cultured in 25°C and split them into 25°C continuous culturing
588 and 15°C condition. The cells were collected at 0, 12, 24 and 48 h after splitting and total RNAs
589 were extracted. cDNA libraries were sequenced and RPKMs for each coding region of the gene
590 were quantified using the Hiseq 2000 (Illumina, San Diego, CA). The raw RPKM values used for

591 calculation were 54.2, 50.0, 30.8 and 35.4 (0, 12, 24 and 48 h, respectively) in 25°C condition, and
592 37.7, 66.4, 77.4, 92.8 (0, 12, 24 and 48 h, respectively) in 15°C condition. The results of
593 semi-quantitative PCR analyses of the EhDES15 gene were shown in the inset. The gene for Actin3
594 was shown as an internal control.
595

596 **Fig. 5. Fatty acid composition and *desB* mRNA level in the *Synechocystis* transformant**

597 Fatty acid compositions (in mol%) were measured from the *Synechocystis* WT and transformant
598 cells grown at 30°C (A, B) or at 22°C for 1 d after cultured under 30°C (C, D). Gas chromatogram
599 focusing on C_{18} fatty acid species in WT cells (red) and in cells expressing EhDES15 (blue) were
600 shown. Each chromatogram is a representative data among three independent experiments. The
601 asterisk indicates a peak for siloxane contamination. Calculation of the mol% of each FAME among
602 total fatty acids in cells grown in 30°C (B) and 22°C (D). White bars, WT cells; black bars,
603 transformant cells. N.D., not detected. Error bars represent the means ± SD for three experiments
604 using independent clones. Levels of the *desB* mRNA in *Synechocystis* WT and transformant cells
605 were quantified using semi-quantitative PCR (E) and quantitative real-time PCR analysis (F).
606

607 **Fig. 6. Fatty acid composition of *desB*-disruptant *Synechocystis***

608 Fatty acid compositions (in mol%) focusing on C_{18} species were measured from the *desB*-disruptant
609 *Synechocystis* (red) and EhDES15-expressing *Synechocystis*-lacking *desB* gene (blue) (A). Each
610 chromatogram is a representative data among three independent experiments. The mol% of each
611 FAME among total fatty acids was calculated (B). White bars, *desB*-disruptant cells; black bars,
612 EhDES15-expressing cells-lacking *desB* gene. Asterisks 1 and 2 indicate peaks for siloxane and
613 1-octadecanol contamination, respectively. White bars, WT cells; black bars, transformant cells.
614 N.D., not detected. Error bars represent the means ± SD for three experiments using independent
615 clones.

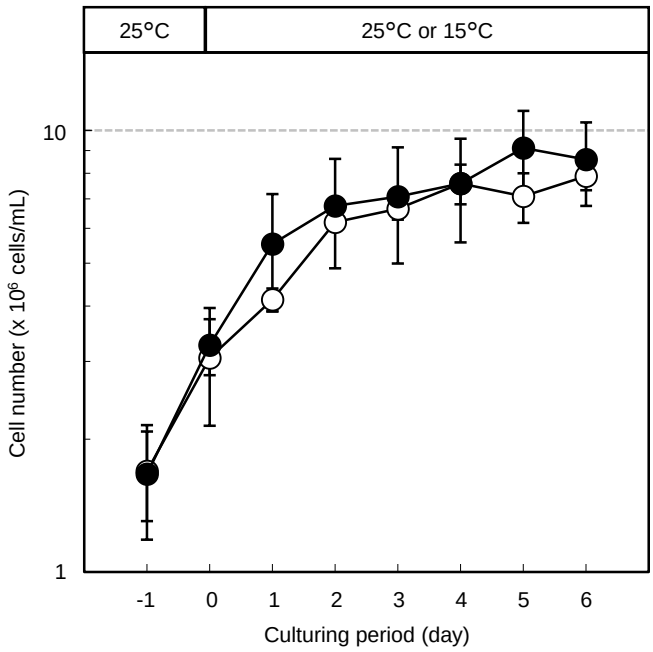


Fig. 1. Growth of *E. huxleyi* cells under 25°C and 15°C conditions

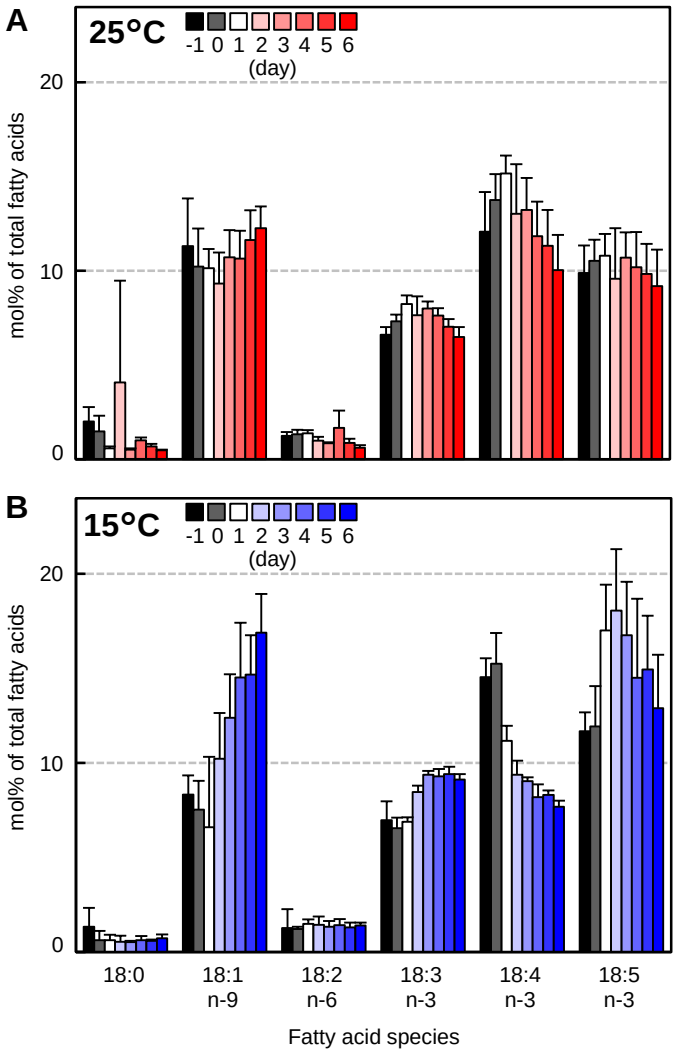


Fig. 2. C_{18} fatty acid composition of *E. huxleyi*

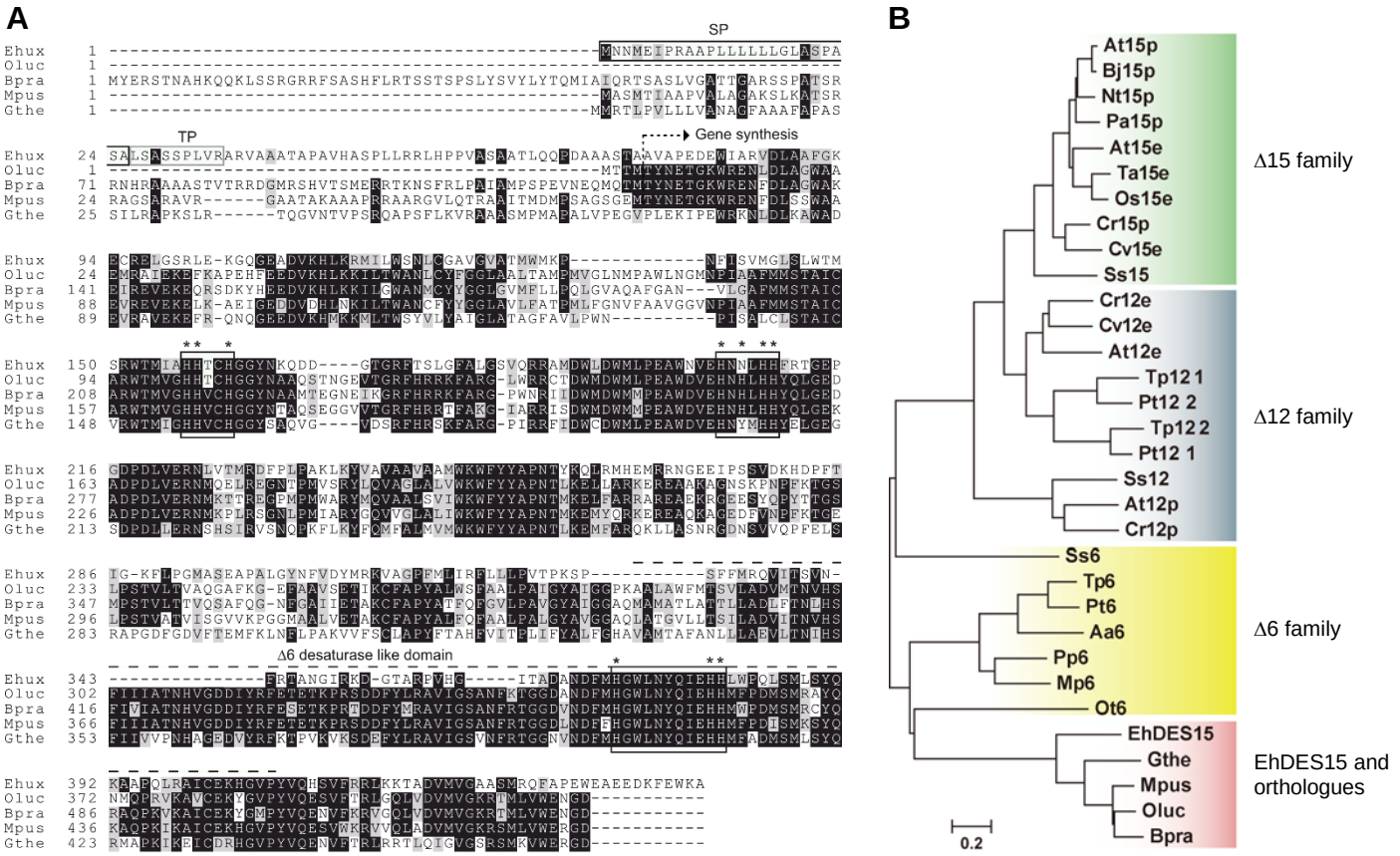


Fig. 3.
Phylogenic analysis of desaturases of microalgae and plants

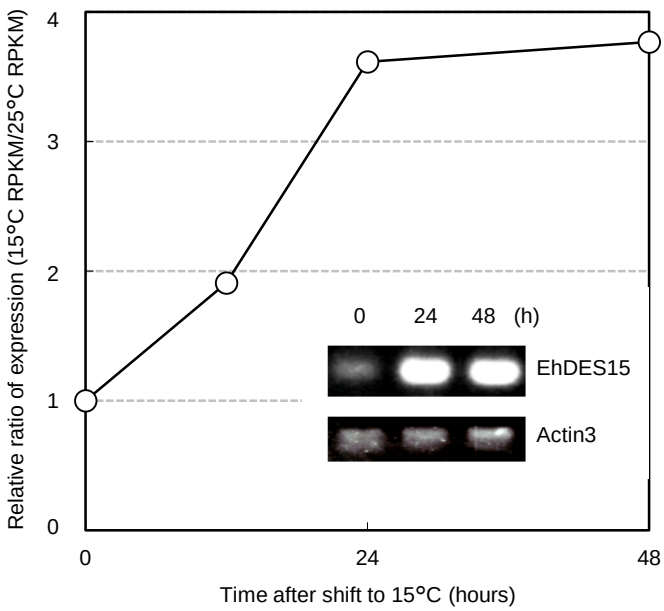


Fig. 4. Accumulation of the EhDES15 gene transcript at low temperature.

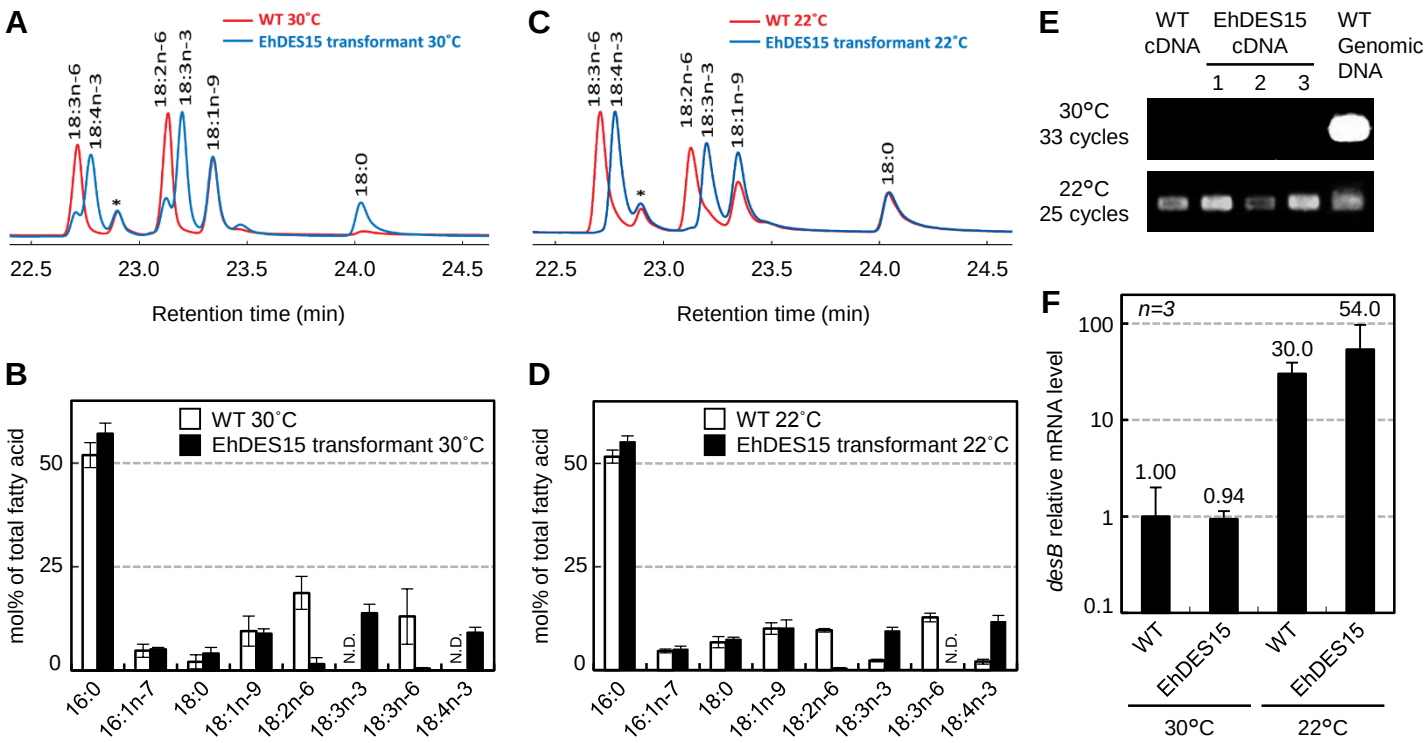


Fig. 5. Fatty acid composition and *desB* mRNA level in the *Synechocystis* transformant

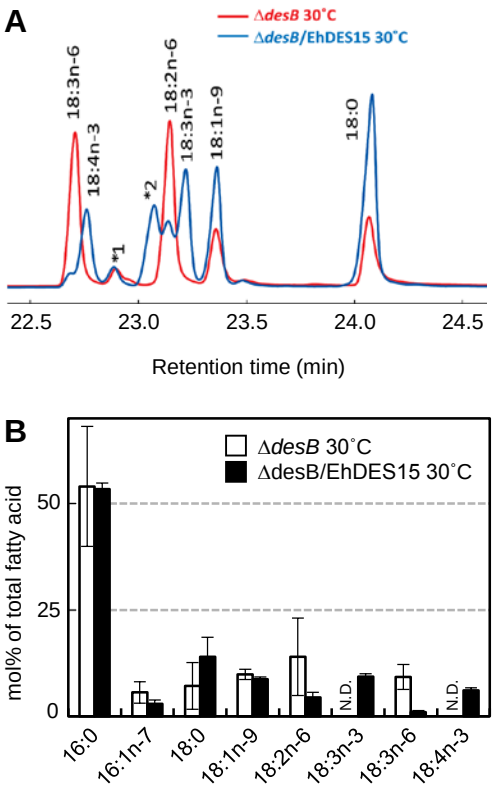


Fig. 6. Fatty acid composition of *desB*-disruptant *Synechocystis*

Supplemental Table 1. Predicted proteins involved in unsaturation of fatty acids in *E. huxleyi*

Proteins were identified from the *E. huxleyi* genome based on homology to typical acyl-lipid desaturase families, including the Δ9-, Δ12-, Δ15- and Δ6-fatty acid desaturases.

Genbank accession number	Length	Closest match on GenBank (identity %)	Homology
EOD33120	254	Δ9 desaturase, *Anaeromyxobacter* sp. Fw109-5 (39%)	Δ9
EOD14752	223	Fatty acid desaturase, *Hahella chejuensis* KCTC 2396 (42%)	Δ9
EOD23549	338	Δ9 desaturase, *Phaeodactylum tricornutum* (55%)	Δ9
EOD31979	400	Fatty acid desaturase, *Alcanivorax borkumensis* SK2 (17%)	Δ12
EOD20437	406	Fatty acid desaturase, *Nostoc punctiforme* PCC 73102 (16%)	Δ12
EOD11132	383	Fatty acid desaturase, *Roseovarius* sp. 217 (23%)	Δ12
EOD23653	370	Fatty acid desaturase, *Nostoc punctiforme* PCC 73102 (17%)	Δ12
EOD16139	373	Fatty acid desaturase, *Prochlorococcus marinus* CCMP1375 (50%)	Δ12 and Δ15
EOD25242	391	Δ12 desaturase, *Caenorhabditis elegans* (19%)	Δ12 and Δ15
EOD26922	418	Microsomal desaturase, *Acanthamoeba castellanii* (37%)	Δ12 and Δ15
EOD07051	386	Microsomal desaturase, *Acanthamoeba castellanii* (36%)	Δ12 and Δ15
EOD29061	397	Hypothetical protein, *Caenorhabditis briggsae* (21%)	Δ12 and Δ15
EOD23700	332	Fatty acid desaturase, *Caenorhabditis elegans* (18%)	Δ15
EOD23435	219	ω13 desaturase, *Chlamydomonas reinhardtii* (33%)	Δ6
EOD07790	76	Δ8 desaturase, *Leishmania braziliensis* (49%)	Δ6
EOD40828	358	Hypothetical protein, *Ostreococcus lucimarinus* CCE9901 (36%)	Δ6
EOD06182	115	Fatty acid desaturase, *Myxococcus xanthus* DK 1622 (49%)	Δ6
EOD40666	448	Fatty acid desaturase, *Ostreococcus lucimarinus* CCE9901 (29%)	Δ6

Supplemental Table 2. Compositional changes in fatty acids at 15°C in *E. huxleyi*

Values indicate the average of triplicate experiments for mol% of total fatty acids. Data represent the means ± SD.

Culturing temperature	25°C		15°C					
Time (*days*)	-1	0	1	2	3	4	5	6
Saturated fatty acid								
14:0	19.4±2.7	21.9± 1.1	18.4±1.4	17.1±3.4	16.8±2.7	15.6±0.7	15.8±2.1	15.2±1.1
16:0	8.6±0.8	7.0± 1.7	7.5±0.9	6.5±1.3	5.6±0.5	5.6±0.4	5.6±0.3	6.1±0.3
18:0	1.3±0.7	0.6± 0.5	0.6±0.3	0.5±0.3	0.5±0.1	0.6±0.2	0.6±0.1	0.7±0.2
22:0	0.5±0.0	0.4± 0.1	0.7±0.1	1.1±0.3	1.5±0.4	2.4±1.1	2.5±1.0	2.9±0.9
24:0	0.3±0.1	0.2± 0.2	0.1±0.0	0.0±0.0	0.0±0.0	0.4±0.6	0.4±0.7	0.5±0.8
Mono-unsaturated fatty acid								
$16{:}1^{\Delta 9}$	1.6±0.1	1.7± 0.4	1.5±0.2	1.3±0.2	1.0±0.2	0.9±0.4	0.8±0.3	0.7±0.5
$18{:}1^{\Delta 9}$	8.3±0.4	7.5± 1.5	6.6±3.7	10.2±2.4	12.4±2.3	14.5±2.9	14.7±2.1	16.9±2.0
$24{:}1^{\Delta 15}$	0.2±0.0	0.2± 0.0	0.3±0.0	0.4±0.1	0.4±0.2	0.2±0.1	0.2±0.1	0.2±0.1
Poly-unsaturated fatty acid								
$18{:}2^{\Delta 9,12}$	1.3±0.2	1.2± 0.1	1.5±0.2	1.4±0.5	1.3±0.3	1.4±0.3	1.3±0.2	1.4±0.2
$18{:}3^{\Delta 9,12,15}$	7.0±1.1	6.5± 0.6	6.9±0.2	8.5±0.4	9.4±0.2	9.3±0.4	9.4±0.4	9.1±0.3
$18{:}4^{\Delta 6,9,12,15}$	14.5±1.9	15.2± 1.6	11.2±0.8	9.4±0.8	9.0±0.2	8.2±0.7	8.3±0.2	7.7±0.3
$18{:}5^{\Delta 3,6,9,12,15}$	11.7±2.6	11.9± 2.1	17.0±2.4	18.1±3.2	16.8±2.8	14.5±4.2	14.9±2.8	12.9±2.8
$20{:}2^{\Delta 11,14}$	0.1±0.1	0.4± 0.3	0.4±0.3	0.6±0.6	0.2±0.1	1.5±2.2	0.7±0.8	1.1±1.3
$20{:}4^{\Delta 5,8,11,14}$	0.1±0.0	0.1± 0.1	0.2±0.0	0.2±0.0	0.2±0.1	0.3±0.3	0.2±0.0	0.2±0.1
$20{:}5^{\Delta 5,8,11,14,17}$	0.8±0.1	0.7± 0.4	0.8±0.5	0.6±0.4	0.5±0.3	0.4±0.3	0.4±0.1	0.5±0.0
$22{:}5^{\Delta 7,10,13,16,19}$	0.5±0.1	0.5± 0.1	0.5±0.1	0.4±0.1	0.4±0.1	0.4±0.2	0.5±0.1	0.5±0.1
$22{:}6^{\Delta 4,7,10,13,16,19}$	22.5±0.7	21.3± 2.4	23.6±3.5	21.8±5.3	22.4±3.6	22.5±3.3	22.2±4.5	22.0±3.8
Others not identified	1.5±0.6	2.5± 0.8	2.2±0.7	1.9±1.0	1.4±0.4	1.3±0.3	1.5±0.7	1.4±0.6
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Supplemental Table 3. Compositional changes in fatty acids at 25°C in *E. huxleyi*

Values indicate the average of triplicate experiments for mol% of total fatty acids. Data represent the means ± SD.

Culturing temperature	25°C		25°C					
Time (*days*)	-1	0	1	2	3	4	5	6
Saturated fatty acid								
14:0	11.2 ±4.5	13.7 ±4.8	14.8 ±2.9	13.3 ±4.6	15.7 ±2.3	16.8 ±0.8	17.2 ±0.6	17.8 ±0.4
16:0	10.1 ±0.7	9.1 ±1.1	8.1 ±0.6	8.3 ±0.5	9.0 ±0.3	9.7 ±0.3	9.8 ±0.4	9.9 ±0.3
18:0	2.0 ±0.8	1.5 ±0.8	0.6 ±0.1	4.1 ±5.4	0.5 ±0.1	1.0 ±0.2	0.7 ±0.1	0.5 ±0.0
22:0	0.8 ±0.3	0.7 ±0.2	0.7 ±0.1	1.1 ±0.6	0.8 ±0.1	0.8 ±0.1	0.8 ±0.1	0.9 ±0.1
24:0	0.2 ±0.1	0.2 ±0.2	0.1 ±0.2	0.1 ±0.1	0.1 ±0.2	0.2 ±0.2	0.1 ±0.2	0.1 ±0.1
Mono-unsaturated fatty acid								
$16{:}1^{\Delta 9}$	1.4 ±0.3	1.1 ±0.3	1.0 ±0.3	0.7 ±0.4	0.9 ±0.3	0.8 ±0.2	1.0 ±0.3	1.0 ±0.2
$18{:}1^{\Delta 9}$	11.3 ±2.5	10.2 ±2.0	10.1 ±1.0	9.3 ±1.6	10.7 ±1.5	10.6 ±1.5	11.6 ±1.6	12.3 ±1.2
$24{:}1^{\Delta 15}$	0.3 ±0.3	0.2 ±0.2	0.1 ±0.1	0.1 ±0.1	0.1 ±0.1	0.1 ±0.1	0.1 ±0.1	0.1 ±0.1
Poly-unsaturated fatty acid								
$18{:}2^{\Delta 9,12}$	1.2 ±0.2	1.3 ±0.2	1.4 ±0.2	1.0 ±0.2	0.9 ±0.1	1.7 ±0.9	0.9 ±0.2	0.6 ±0.1
$18{:}3^{\Delta 9,12,15}$	6.6 ±0.4	7.3 ±0.4	8.2 ±0.5	7.6 ±1.0	8.0 ±0.4	7.6 ±0.4	7.0 ±0.4	6.5 ±0.5
$18{:}4^{\Delta 6,9,12,15}$	12.1 ±2.1	13.8 ±1.4	15.2 ±1.0	13.0 ±2.6	13.2 ±1.7	11.8 ±1.8	11.3 ±1.9	10.0 ±1.9
$18{:}5^{\Delta 3,6,9,12,15}$	9.9 ±1.5	10.5 ±1.1	10.8 ±1.2	9.6 ±2.7	10.7 ±1.3	10.2 ±1.9	9.8 ±1.6	9.2 ±1.9
$20{:}2^{\Delta 11,14}$	0.2 ±0.0	0.2 ±0.1	0.2 ±0.1	0.2 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0
$20{:}4^{\Delta 5,8,11,14}$	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0
$20{:}5^{\Delta 5,8,11,14,17}$	0.6 ±0.2	0.5 ±0.3	0.4 ±0.4	0.3 ±0.3	0.3 ±0.3	0.2 ±0.3	0.2 ±0.3	0.2 ±0.3
$22{:}5^{\Delta 7,10,13,16,19}$	0.8 ±0.3	0.6 ±0.2	0.6 ±0.3	0.8 ±0.4	0.8 ±0.3	0.8 ±0.3	0.9 ±0.3	1.0 ±0.4
$22{:}6^{\Delta 4,7,10,13,16,19}$	28.8 ±5.1	27.3 ±4.2	26.5 ±3.3	29.2 ±6.2	27.0 ±3.6	26.3 ±2.4	27.2 ±2.3	28.8 ±2.4
Others not identified	2.4 ±0.7	1.6 ±0.2	1.2 ±0.2	1.3 ±0.7	1.2 ±0.1	1.1 ±0.1	1.1 ±0.1	1.0 ±0.1
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Supplemental Fig. 1. Changes in total fatty acids of *E. huxleyi* under 25°C and 15°C conditions

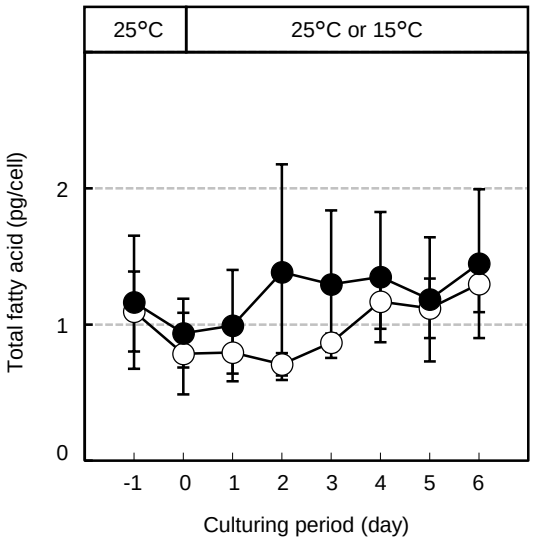
Closed and open circles show total fatty acids levels at 25°C and 15°C, respectively. Each point represents the average for three experiments, and the error bars represent the means ± SD.

Supplemental Fig. 2. Fatty acid composition of *E. huxleyi* (mol%).

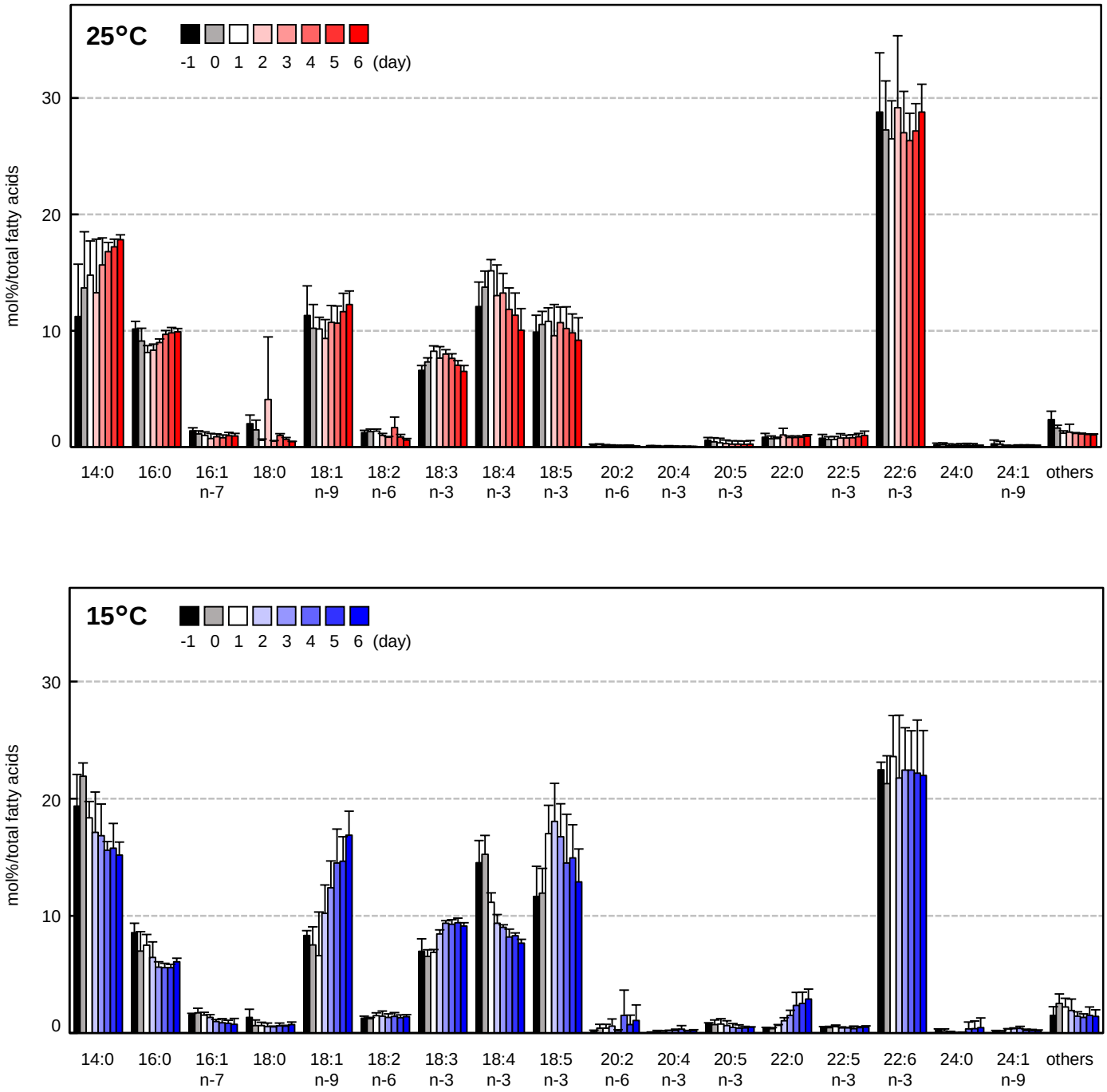
Changes in fatty-acid levels in cells cultured continuously at 25°C (A) and cells transferred from 25°C to 15°C (B). Each bar represents the average for triplicate experiments, and error bars represent the means ± SD.

Supplemental Fig. 3 Fatty acid composition of *E. huxleyi* (pg/cell)

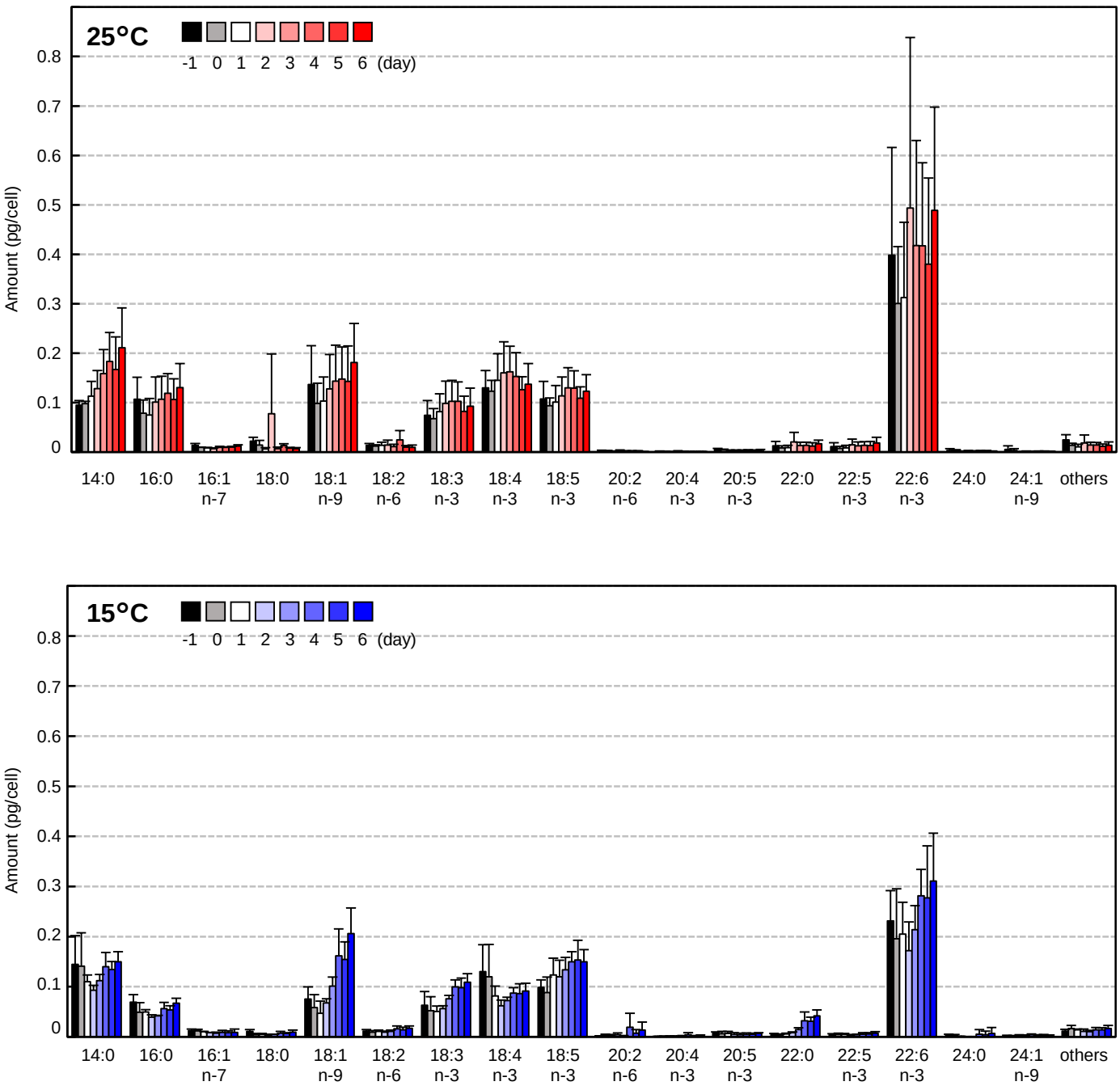
Changes in absolute amounts of each fatty acid species in cells cultured continuously at 25°C (A) and cells transferred from 25°C to 15°C (B). Each bar represents the average for triplicate experiments, and error bars represent the means ± SD.



Supplemental Fig. 1.
Changes in total fatty acid of *E. huxleyi* under 25°C and 15°C conditions



Supplemental Fig. 2. Fatty acid composition of *E. huxleyi* (mol%)



Supplemental Fig. 3. Fatty acid composition of *E. huxleyi* (pg/cell)