Studies on Distribution and Synthetic Pathway of Octadecapentaenoic Acid, a Characteristic Polyunsaturated Fatty Acid, in Microalgae

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Abbreviations

12:0	dodecanoic acid; lauric acid
14:0	tetradecanoic acid; myristic acid
15:0	pentadecanoic acid
16:0	hexadecanoic acid; palmitic acid
16:1n-7	16:1Δ9; *cis*-Δ9-hexadecenoic acid
16:1n-9	16:1Δ7; *cis*-Δ7-hexadecenoic acid
16:1n-13t	*trans*-Δ3-hexadecenoic acid
16:2n-6	16:2Δ7,10; *cis*-Δ7,10-hexadecadienoic acid
16:3n-3	16:3Δ7,10,13; *cis*-Δ7,11,13-hexadecatrienoic acid
16:4n-3	16:4Δ4,7,10,13; *cis*-Δ4,7,10,13-hexadecatetraenoic caid
18:0	octadecanoic acid; stearic acid
18:1n-9	18:1Δ9; *cis*-Δ9-octadecenoic acid; oleic acid
18:1n-7	18:1Δ11; *cis*-Δ11-octadecenoic acid; vaccenic acid
18:2n-6	18:2Δ9,12; *cis*-Δ9,12-octadecadienoic acid; α-linoleic acid
18:3n-3	18:3Δ9,12,15; *cis*-Δ9,12,15-octadecatrienoic acid; α-linolenic acid
18:3n-6	18:3Δ6,9,12; *cis*-Δ6,9,12-octadecatrienoic acid; γ-linolenic acid
18:4n-3	18:4Δ6,9,12,15; *cis*-Δ6,9,12,15-octadecatetraenoic acid; stearidonic acid
18:5n-3	18:5Δ3,6,9,12,15; *cis*-Δ3,6,9,12,15-octadecapentaenoic acid
20:0	eicosanoic acid
20:1n-9	20:1Δ11; *cis*-Δ11-eicosaenoic acid
20:2n-6	20:2Δ11,14; *cis*-Δ11,14-eicosadienoic acid

20:3n-6	20:3Δ8,11,14; *cis*-Δ8,11,14-eicosatrienoic acid
20:4n-3	20:4Δ5,8,11,14; *cis*-Δ5,8,11,14-eicosatetraenoic acid; arachidonic acid
20:5n-3	20:5Δ5,8,11,14,17; *cis*-Δ5,8,11,14,17-eicosapentaenoic acid; EPA
22:0	docosanoic acid
22:5n-3	22:5Δ7,10,13,16,19; *cis*-Δ7,10,13,16,19-docosapentaenoic acid;
22:6n-3	22:6Δ4,7,10,13,16,19; *cis*-Δ4,7,10,13,16,19-docosahexaenoic acid; DHA
24:0	tetracosanoic acid
24:1n-9	24:1Δ13; *cis*-Δ13-tetracosaenoic acid
26:0	hexacosanoic acid
ACP	acyl carrier protein
CCMP	culture collection of marine phytoplankton
CoA	coenzyme A
Cyt b_5	cytochrome b_5
DGDG	digalactosyl diacylglycerol
ER	endoplasmic reticulum
FAME	fatty acid methyl ester
GC-FID	gas chromatography equipped with flame ionization detector
GC-MS	gas chromatography equipped with mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MGDG	monogalactosyl diacylglycerol
NIES	National Institute for Environmental Studies (the strain number of NIES culture collection)
OD_{730}	optical density at 730 nm

ORF	open reading flame
PC	phosphatidylcholine
PCC	strain number of Pasteur Culture Collection
PUFA	polyunsaturated fatty acid
SQDG	sulfoquinovosyl diacylglycerols
Tris	tris(hydroxymethyl)aminomethane

Abstract

Polyunsaturated fatty acids (PUFAs) are important lipid components for determining the membrane fluidity of cell membranes. Fatty acid desaturases play a key role for regulating physical property of membrane by introducing carbon double bonds into fatty acids, especially in the case of cold stress conditions. Non-photosynthetic organisms including human beings are not able to synthesize some PUFA species but they are essential for surviving. Thus, the organisms are depending on the acquisitions of the fatty acids for ingredients of the dietaries. Some species of microalgae produce a PUFA, namely octadecapentaenoic acid (*cis*-Δ3,6,9,12,15-18:5, hereafter referred to as 18:5n-3), which is C_{18} fatty acid containing 5 *cis*-type carbon double bonds. In contrast to model organisms: *Arabidopsis thaliana* synthesized 18:3n-6 and some cyanobacteria produced 18:4n-3 as the most highly unsaturated C_{18} fatty acid, the 18:5n-3 production is observed in a few species of microalgae e.g. haptophytes, dinoflagellates and heterokontophytes as secondary plants which origin of chloroplast is originated from red algal symbionts and prasinophytes from chlorophytes. Although other PUFAs such as eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) have already well-studied on their metabolic pathway, distribution of microalgal species and its commercial production, the function and synthetic pathway for 18:5n-3 are less characterized. In this study, I tried to determine 18:5n-3 distribution in prasinophytes and investigate the desaturase gene(s) involved in 18:5n-3 biosynthesis.

[Since this paragraph contains an explanation of unpublished data, it is concealed until the paper is published.]

For further physiological analysis of 18:5n-3 and characterization of desaturase gene, a unicellular marine phytoplankton, *E. huxleyi* was used. In *E. huxleyi* cells, 18:5n-3 is accumulated as an acyl-group of glycerolipid species, such as monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) (Bell and Pond, 1996) in thylakoid membrane of plastid. I tried to investigate the genes contributing 18:5n-3 production in *E. huxleyi*. I firstly grew the *E. huxleyi* cells at 25˚C and transferred and further incubated at 15˚C because the desaturation of membrane lipid generally occurs in low temperature condition. The cells were harvested every day and the fatty acid compositions were determined. After 2 d cultured at 15˚C, the content of 18:5n-3 in total fatty acid was significantly increased from 11.9

mol% at 0 d to 18.1 mol%, whilst 18:4 content was declined from 15.2 mol% to 9.4 mol%. Beside these two PUFAs, 18:3n-3 was also increased from 6.5 mol% to 8.5 mol%. After 6 d cultured at 15°C, the content of a mono-unsaturated fatty acid, 18:1n-9 was increased at the most highly induced in the low temperature condition. These results suggested that 18:5n-3 might be synthesized from 18:4 via desaturation reaction induced at low temperature. According to results published by Bell and Pond (1996), desaturases involved in 18:5n-3 synthesis are expected to be localized in thylakoid membrane in the plastid. Plants possess two types of desaturases: one is localized in the plastid and receive an electron from ferredoxin, the other is in endoplasmic reticulum (ER) membrane and cytochrome b_5 (Cytb_5) works as an electron donor. According to the genomic sequence of *E. huxleyi*, I screened 18 desaturase genes by a homology to well-known plant type desaturases and by the absence of Cytb_5 domain. A gene among 18 candidates, EOD40666 (Genbank accession number, Read et al., 2013) has a homology to Δ6 desaturase-like domain and possesses a putative signal peptide at N-termini. The orthologues protein was conserved in both genomes of *Ostreococcus* and *Micromona*s as 18:5n-3 producers. Its gene expression was increased up to 2.5 times higher at 15°C compared at 25°C. Heterologous expression analysis of the gene in the wild-type cells of the cyanobacterium *Synechocystis* sp. PCC 6803 revealed that the accumulation of PUFAs possessing a carbon double bond at Δ15 position: 18:3n-3 and 18:4n-3. EOD40666 expression complemented the phenotype of Δ*desB Synechocystis* mutant lacking the gene for native Δ15 desaturase. Because *Synechocystis* originally harbors only ferredoxin type desaturase, these results suggested that the gene EOD40666 encoded a novel Δ15 fatty acid desaturase involved in PUFAs biosynthesis in *E. huxleyi* and is predicted to be functioned in plastid.

General Introduction

Membrane fluidity and unsaturated fatty-acid

For all living organisms, temperature is a major environmental factor that is fluctuating diurnally, seasonally and also according to their habitats. They are able to sense transient changes in temperature and to respond to the changes for adjusting their cell metabolism. The regulation of fluidity of biomembranes: plasma membrane, endoplasmic reticulum (ER) membrane or thylakoid membrane in plants, is a crucial process for acclimating to the change in temperature (Fig 1; Russell, 1984; Farrell and Rose, 1967). Many studies focused on the effects of low temperature and investigated clearly that membrane fluidity is decreases by the downward shift in temperature. On the other hand, high temperature stress causes membrane fluidization, which causes disintegration of lipid bilayer. It is clear that both low and high temperature conditions modulate membrane fluidity (Quinn and Williams, 1983; Los and Murata, 2004).

It has been well-characterized that the content of unsaturated fatty acid in the membrane glycerolipids changes in response to the changes in growth temperature. The degree of unsaturated fatty acids in membrane lipids plays a key role in the regulation of membrane fluidity and in the maintenance of the activity of membrane-bound proteins correlated with the temperature shift (Raison, 1973). When a *cis*-type double-bond is introduced into the acyl chain of saturated fatty acid to cause mono-unsaturated fatty acid, the straight acyl chain is bent at the position of the *cis*-type double bond, which alters the distance between surrounding lipid molecules to increase in the membrane fluidity. Additionally, in the downward shift of temperature, the appropriate increase of unsaturated fatty acids in the membrane lipids

decreases the temperature for the transition from the gel to the liquid-crystalline phase to provide cells' chilling tolerance. Unsaturated fatty acids which harbors more than two carbon-double bonds are generally called as a poly-unsaturated fatty acids (PUFAs). PUFAs are synthesized generally by most of organisms. Although some PUFAs are essential for surviving of the organisms, non photosynthetic organisms including human beings are not able to synthesize the ω3- and ω6-PUFAs and are dependent on ingredients of dietaries (Riediger et al., 2009). α-linoleic acid (18:3n-3; 18:3Δ9,12,15), docosahexaenoic acid (22:6n-3; 22:6Δ4,7,10,13,16,19; DHA) and eicosapentaenoic acid (20:5n-3; 20:5Δ5,8,11,14,17; EPA) are well-known ω3-PUFAs and are essential nutrients for human diet.

Fatty acid biosynthesis in microalgae

The biosynthetic pathway for unsaturated fatty acid production in plants and microalgae has been studied by the characterization of mutants inactivated the genes involved in the unsaturated fatty-acid synthesis. The unsaturated fatty acids are generally produced from saturated fatty acids catalyzed by so-called "fatty acid desaturase" (Fig 2). Fatty acid desaturases convert a single bond between two carbon atoms (C-C) to a *cis*-type double bond (C=C) in acyl group in the fatty acid. Although desaturation reactions are mostly occurred in the acyl-chain of glycerolipid catalyzed by acyl-lipid fatty acid desaturase, the first desaturation reaction introduced into the saturated fatty acyl chain is often occurred in acyl-Coenzyme A (CoA) and acyl-acyl carrier protein (ACP). The Δ9 acyl-ACP fatty acid desaturase from a green alga, *Chlamydomonas reinhardtii*, catalyzes the desaturation reaction converting the saturated acyl-ACP, 18:0-ACP, to monounsaturated acyl-ACP, 18:1n-9-ACP. Then 18:1n-9-ACP is integrated into galactolipid species in thylakoid membrane in chloroplast, i.e. mono- and di-galactosyl diacylglycerol (MGDG or DGDG), by acyl-ACP transferase (Browse and

Somerville, 1991). The Δ12 and Δ15 acyl-lipid desaturases are responsible for the synthesis of 18:3n-3 from 18:1n-9 in *C. reinhardtii* (Sato et al., 1997; Iba et al., 1993). In eukaryotic photosynthetic organisms, there is an alternative pathway to produce unsaturated C_{18} fatty acid species in ER membrane namely eukaryotic pathway. The unsaturated acyl-groups of glactolipids synthesized in the chloroplast are then acyl-transferred into phospholipids in ER membrane and further elongated or desaturated by ER-located enzymes. *C. reinhardtii* also possesses Δ12 and Δ15 acyl-lipid fatty acid desaturases predicted to be localized in ER membrane. These two types of desaturases localized both in ER and in chloroplast are structurally similar in the catalytic domain consisting of three conserved histidine motifs, but they can be separated by their electron donors. Desaturases located in ER usually contain a Cyt b_5 domain accepting electrons from NADH via cytochrome reductase and functioning as an electron donor for the desaturation reaction, whereas chloroplast-localized desaturases do not have it and the electron donor is ferredoxin. For example, both cyanobacteria *Synechocystis* sp. PCC6803 (hereafter referred to as *Synechocystis*) and the moss *Physcomitrella patens* possess Δ6 desaturase and can synthesize 18:4Δ6,9,12,15 (stearidonic acid; 18:4n-3). The desaturase in *Synechocystis* utilizes ferredoxin, whilst the desaturase in *P. patens* is located on ER and contains a Cyt b_5 domain (Girke et al., 1998). The chloroplast-localized desaturases are often characterized by mutants which affected fatty acid compositions. On the other hand, the ER-localized desaturases are well-investigated by heterologously functional expression in the cells of *Saccharomyces cerevisiae* which easily uptake a variety of fatty acids and incorporate into their membrane lipids. For example, the synthetic pathways for 22:6n-3 and 20:5n-3 in a diatom, *Thalassiosira pseudonana*, are investigated by functional expression in the yeast cells and these fatty acids are synthesized by eukaryotic pathway consisting of the ER-located Δ4 or Δ5 acyl-lipid desaturase and elongation systems (Fig 3; Tonon et al., 2005).

PUFAs distribution and biosynthesis in microalgae

Many previous studies have shown the fatty acid composition of numerous number of photosynthetic organisms (Nichols et al., 1965; Allen et al., 1964; Giroud et al., 1988) and suggested the trend in fatty acid composition depending on a specific group of phylogenic position. Therefore, some fatty acid species are used as a biomarker for indicating the organisms. For example, some species of microalgae produce a PUFA, namely octadecapentaenoic acid (18:5Δ3,6,9,12,15; 18:5n-3), which is C_{18} fatty acid containing 5 *cis*-type carbon double bonds. Comparing to 18:3n-3 synthesized by *Arabidopsis thaliana* and 18:4n-3 by some cyanobacteria and *P. patens* as the most highly unsaturated C_{18} fatty acid, the 18:5n-3 production is observed in a few species of microalgae. Until 1970's, 18:5n-3 has been thought to be a biomarker for dinoflagellates. Recent researches reveal that other microalgal groups can also synthesize 18:5n-3, 11 genera of dinoflagellates, 6 of haptophytes, 2 of heterokontophytes and 4 of prasinophytes (Phylum Chlorophyta) have been found to produce 18:5n-3 (Table 1). Former three groups of microalgae are secondary plants and prasinophyte is green algae (see following paragraph: primary plant and secondary plant in general introduction). Another characteristic PUFA detected in a specific microalga is 16:4Δ4,7,10,13 (16:4n-3). It has been detected mostly in green algae, such as *Chlamydomonas*, *Chlorella* and prasinophytes (Dunstan et al., 1992; Lang et al., 2011; Chia et al., 2013) and is often used as a biomarker for these algae. *Chlamydomonas* is known to produce another PUFA, 18:4Δ5,9,12,15, which structure is quite unique because carbon double-bonds of PUFAs are normally introduced at intervals of three carbons, e.g. 18:3Δ9,12,15 or 18:4Δ6,9,12,15. Comparing these C_{16} or C_{18} PUFAs found in a certain group of microalgae, 20:5n-3 and 22:6n-3 are widely distributed over the bacteria and various supergroups of eukaryote (Lang et al., 2011). Classification of microalgae by statistical

analysis using their fatty acid composition has been demonstrated (Temina et al., 2007; Lang et al., 2011).

The difference on the composition and distribution of PUFAs should be caused by the expression of genes for of specific fatty-acid desaturases. For instance, *C. reinhardtii* has four genes encoding plastid-located fatty-acid desaturase: Δ4, Δ7, Δ10 and Δ13 fatty acid desaturases, involved in 16:4n-3 biosynthesis (Zäuner et al., 2012; Kunst et al., 1989; Sato et al., 1997; Iba et al., 1993). These genes are characterized using a mutant strain of *C. reinhardtii* which abolished the activity to introduce each desaturation reaction to produce unsaturated C_{16} fatty-acid derivatives. Using model plants or microalgae, fatty acid metabolisms including PUFAs biosynthesis are well-studied because their genomic sequences are available and they can be modified genetically to construct the mutant lines. However, PUFA metabolisms in non-model plants and microalgae, i.e. 18:5n-3 biosynthetic pathway, are not well-understood yet because the distribution of 18:5n-3 is very limited in a few species of microalgae which are not able to be genetically modified.

Primary plants and secondary plants

All photosynthetic eukaryotes, namely microalgae, macroalgae and vascular plants, can be divided into two different groups, i.e. primary plants and secondary plants. Primary plants contain three major groups: green plants, red algae and glaucophytes, which had been evolved by the primary endosymbiosis event to acquire the chloroplast (Margulis, 1971; Martin and Kowallik, 1999). There are two major groups in green plants. One is a group of streptophytes consinting of land plants and the other is a group of chlorophytes including green algae (Leliaert et al., 2012). According to the fossil record, green algae and red algae were evolved in 930 million years ago, and land plants was split from a green algal branch in 510 million years ago

(Berney and Pawlowski 2006). The gourp of green plants is the most abundant and diverse group in terrestrial habitats. Prasinophytes (Chlorophyta) are thought to be an ancient group of green algae and to form a paraphyletic lineage, which had been evolved by shortly after the primary endosymbiosis event (Kantz et al., 1990; Steinkötter et al., 1994; Nakayama et al., 1998; Leliaert et al., 2011). It is quite important to study on prasinophytes in order to estimate the evolutionary implication of green algae.

In contrast to the diversity of green algae in terrestrial habitats, the marine environments are dominated by red algae and secondary plants. After primary endosymbiosis, some red algae and green plants were engulfed by heterotrophic eukaryote via secondary endosymbiosis to generate secondary plants. (McFadden, 2001). There are six lineages of microalgae categorized into the secondary plants. Haptophytes, dinophytes, heterokontophytes and cryptophytes acquire their plastids from the red algae and euglenids and chlorarachniophytes acquire their plastids from the green algae.

Interestingly, when focusing on PUFAs compositions in the primary and secondary plants, 18:5n-3 was found only in prasinophytes, a primary plant belonging to green algae, and haptophytes, dinophytes and heterokontophytes which the origin of chloroplast are originated from red algal endosymbionts. Whereas, other ω3 PUFAs: EPA and DHA are synthesized in a various group of eukaryotic microalgae including diatoms which the origin of chloroplast is originated from red algal symbionts (Lang et al., 2011; Tonon et al., 2002) and even in bacteria. Almost all bacteria cannot synthesize any PUFAs in the cells but a few of marine bacteria are known to possess an alternative enzyme to synthesize DHA, which differs from a typical elongation-desaturation PUFAs synthetic pathway (Yazawa, 1996; Amiri-Jami and Griffiths, 2010).

[Since this paragraph contains an explanation of unpublished data, it is concealed until the paper is published.]

In the Chapter II of this Ph.D thesis, using a haptophyte *E. huxleyi*, I tried to identify the enzymes involved in PUFAs biosynthesis, especially in 18:5n-3 biosynthesis. I observed the compositional changes in unsaturated fatty acids in the cells of *E. huxleyi* grown at low temperature conditions. With reference to the genomic sequence of *E. huxleyi* (Read et al., 2013), I finally characterized a novel plastidial Δ15 fatty acid desaturase, which produces 18:3n-3 and 18:4n-3 from 18:2n-6 and 18:3n-6, respectively, by heterologous expression of the gene in a cyanobacterium, *Synechocystis*. The desaturase I identified was responsible for 18:5n-3 because two products of the desaturase were both intermediates of 18:5n-3 biosynthesis. Furthermore, the orthologues were found in genomes of other 18:5n-3-producers, *Ostreococcus* and *Micromonas*. As results of the Chapter I and II, I discussed the evolutionary implication of 18:5n-3 biosynthesis of microalgae and the importance of identifications of plastid-located fatty acid desaturases involved in PUFAs biosynthesis in the plastid.

Chapter I

Distribution of fatty acid composition in prasinophytes (Chlorophyta)

Introduction

[Since this chapter contains an explanation of unpublished data, it is concealed until the paper is published.]

Materials and Methods

[Since this chapter contains an explanation of unpublished data, it is concealed until the paper is published.]

Results

[Since this chapter contains an explanation of unpublished data, it is concealed until the paper is published.]

Discussion

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Chapter II

Identification of a fatty acid desaturase involved in PUFA biosynthesis in *E. huxleyi*

Introduction

In the Chapter II, I try to characterize the genes for fatty acid desaturases involved in PUFAs biosynthesis using a microalgae, coccolithophorid (classified in Phylum Haptophyta) *E. huxleyi*. This microalga is known to produce coccolith, often form blooms in the early summer until mid-summer in the ocean, thought to contribute to the global carbon cycle (Orr et al., 2005; Fukuda et al., 2011; Winter et al., 1994). 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. Among haptophytes, *E. huxleyi* is used for numerous comparative physiological studies, since it is a contemporary species and grows well in laboratory cultures (Paasche, 2001). In accordance with lipid analyses, *E. huxleyi* accumulates very long chain ketones, namely alkenone (Volkman et al., 1986) and PUFAs including 18:5n-3, 20:5n-3 and 22:6n-3 (Bell and Pond, 1996). 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 (Volkman et al., 1998). 18:5n-3 has been identified in some species of red plastid-derived secondary plant and prainophytes (Phylum Chlorophyta) (see Chapter I for more about 18:5n-3 destribution in prasinophytes). Among the haptophytes (besides *E. huxleyi*), *Isochrysis* sp. (Renaud et al., 1995), *Chrysochromulina polylepis* (John et al., 2002), *Crystallolithus hyalinus* (Volkman et al., 1981), *Hymenomonas elongate* (Viso and Marty et al., 1993), *Prymnesium saltans* and *Coccolithophora* sp. (Lang et al., 2011) produce 18:5n-3 (Table 1). In *E. huxleyi*, 18:5n-3 is attached to glycolipids, such as mono- and di-galactosyl diacylglycerol (MGDG and DGDG) (Bell and Pond, 1996), indicative of thylakoid membrane localization and of a relationship with photosynthetic functions and machinery stability. In

contrast to 20:5n-3 and 22:6n-3 which caused by the ER-located front-end desaturase enzymes, biosynthetic pathway for 18:5n-3 is still less studied. Sayanova and co-workers (2011) hypothesized that 18:5n-3 biosynthesis in *E. huxleyi* is catalyzed by the Δ3 fatty acid desaturase introducing carbon-double bond into 18:4n-3 or by the β-oxidation reaction of 20:5n-3. But nobody has demonstrated the reaction yet.

As previously described in general introduction, fatty acid desaturases can be divided into two kinds of desaturase by their localizations: plastid-located and ER-located desaturases (see general introduction). There are many studies on the identification of both types of fatty acid desaturase in various organisms. In model plants or model microalgae, identification of fatty acid desaturase is classically performed by the phenotype of the mutant lacking the productivity of certain unsaturated fatty acid. For example, in a cyanobacterium *Synechocystis*, totally four acyl-lipid desaturases were identified by the mutant disrupting each gene (Sakamoto et al., 1994a; Wada et al., 1990; Sakamoto et al., 1994b; Reddy et al., 1993). However for the non-model plants or non-model microalgae, it will be difficult to characterize the genes because of their inconvenience for laboratory use, e.g. difficulties of culturing, transformation or less genomic information. Therefore, in order to characterize the desaturase genes from non-model organisms, heterologous expression of the genes is often performed. Because of the benefit for human diet or the industrial application of PUFAs, the genes for Δ5 desaturase and Δ4 desaturase, which catalyze the final desaturation reaction to produce 20:5n-3 and 22:6n-3, respectively, have been often isolated from various microalgae and characterized by a heterologous expression using yeast cells. This approach is enabled by the feature of ER-located front-end desaturase such as Δ5 and Δ4 desaturases, that possess an intramolecular Cyt b_5 domain to transfer the electron for desaturation reaction. 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. Because the Cyt b_5-containing desaturase functions in heterologously-expressed cells and yeast can uptake various fatty acids and incorporate them into their membrane lipids as substrates for the desaturases, identification of ER-located and Cyt b_5-containing desaturase is often carried out by heterologous expression analysis in the yeast cells. Some desaturases in the haptophytes *E. huxleyi*, *Isochrysis galbana* and *Pavlova salina* were previously characterized using heterologous expression analysis (Fig 3; Sayanova et al., 2011; Pereira et al., 2004; Shi et al., 2012). However, for the characterization of chloroplast-located desaturase by heterologous expression, it is necessary to use a photosynthetic organism as host cells because of requirement of the plastidial Fe_2-S_2 ferredoxin for supply of electron.

In Chapter II of this Ph.D thesis, I report the screening and functional characterization of a novel Δ15 acyl-lipid desaturase from *E. huxleyi* based on heterologous expression in *Synechocystis*. I selected a gene which is predicted to be localized in the plastid and is a candidate of Δ3 desaturase by genomic search of 18:5n-3-producing microalgae including *E. huxleyi* in Chapter I (Table 5). When I expressed the *E. huxleyi* gene encoding a putative plastidial acyl-lipid desaturase homologous to Δ6 desaturase in *Synechocystis*, which was conserved in both genomes of *O. lucimarinus* and *M. pusilla* as 18:5n-3 producer, the cells of the transformants accumulated 18:3n-3 and 18:4n-3 and showed decreased amounts of 18:2n-6 and γ-linolenic acid (18:3n-6). These results suggested that the gene encodes a novel Δ15 desaturase responsible for the synthesis of 18:3n-3 from 18:2n-6 in *E. huxleyi*. In this report, I discuss PUFA metabolism in divergent microalgae and the usability of cyanobacteria as a tool for functional analysis of plastidial desaturases.

Materials and Methods

Organisms and culture conditions

In order to observe the temporal change in fatty acid composition of *E. huxleyi*, cells of *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 (MA-ESM; Danbara and Shiraiwa, 1999). 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 the measurement of 18:5n-3 and 22:6n-3 of haptophycean algae, I stationary cultured totally 28 strains of haptophytes with 50 ml MA-ESM in Erlenmeyer flask under 70 µmol photons m^{-2} s^{-1} illumination at 20°C.

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) (Stanier et al., 1971) 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 extraction and analysis

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. Lipid extraction and compositional analysis of FAMEs were carried out using the method described in Chapter I. To identify 18:3n-3 and 18:4n-3, which were newly synthesized by expression of the desaturase gene in *Synechocystis*, I used GC-MS with electron ionization and compared the retention times and mass spectrums between the standard methyl-esters of 18:3n-3 and 18:4n-3 (Sigma-Aldrich, Tokyo, Japan) and candidate methyl-esters of 18:3n-3 and 18:4n-3 observed in transformant cells.

RNA extraction

For the RNA extraction from *E. huxleyi*, I 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 15°C, 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, I constructed a plasmid for transformation of *Synechocystis* cells. pTCHT2031V (Ishizuka et al., 2006; 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 (Amann and Brosius, 1985), the downstream sequence of the *slr2031* gene and the plasmid backbone from the pUC vector.

I 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 (Read et al., 2013) 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. I 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. I amplified a DNA fragment of the spectinomycin resistance gene using the primers spr_F_BglII (GGAGATCTATCAATTCCCCTGCTCGCGC) and spr_R_BamHI (GGGGATCCTCCCAATTTGTGTAGGGCTT) and pAM1146 (Tsinoremas et al., 1994) 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, I 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 (Sakamoto et al., 1994b) *Synechocystis* by homologous recombination (Williams, 1988). After verifying full segregation of the chromosome by PCR, fatty acid compositions and gene expression were analyzed.

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

Lipid analysis of haptophytes and 18:5n-3 detection in *E. huxleyi*

I cultured *E. huxleyi* cells under 25°C condition and extracted total lipid. After methyl-esterification, lipid composition was analyzed by GC-MS (Fig 8A). As a major component of fatty acid, 14:0, 16:0, 18:1n-9, 18:4n-3 and 22:6n-3 were detected. Sterols and alkenones could be observed. Alkenones were C_{37}-C_{39} long-chain ketones used as a biomarker of haptophycean algae (Volkman et al., 1980). Besides them, several peaks which are thought to be C_{18} fatty acid derivatives were observed in the chromatogram (Fig 8B). By comparing fragmented mass spectra to that of commercial compounds and that reported previously, I concluded that *E. huxleyi* produced totally six species of unsaturated C_{18} fatty acids: 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3 and 18:5n-3. A fragmentation pattern of 18:5n-3 resembled to that in previous study (Fig 8C; Lang, 2007).

28 strains of haptophytes: 17 strains of *E. huxleyi*, 4 strains of *G. oceanica*, 4 strains of *I. galbana* and each one strain of *Tisochrysis lutea*, *Helladosphaera* sp. and *Pleurochrysis carterae*, were cultured in order to compare the productions of 18:5n-3 and 22:6n-3 (Table 6). Under 20°C-cultured condition, except *P. carterae*, all haptophytes produced 18:5n-3. Production of 18:5 was not a common phenotype among haptophytes. Whereas, 22:6n-3 was detected in all haptophytes tested.

Fatty acid composition of *E. huxleyi*

I 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. I withdrew aliquots of the cultures every 24 h

for 6 days and measured the cell density (Fig 9A). 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^{-1}) in the cultures grown at 15°C and 8.6 ± 1.8 × 10^6 (cells ml^{-1}) in the cultures grown at 25°C. I 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 $cell^{-1}$ in the cells grown at 15°C and 25°C, respectively (Fig 9B). 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 10A). When the cultures were transferred to 15°C and incubated for 2 days, I 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 10B). During further cultivation at low temperature, the content of 18:5n-3 gradually decreased to 12.9 ± 2.8 mol% by day 6 (Table 7, 8; 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. 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 (Nishida and Murata, 1996). 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 acid, 22:0 was increased. These changes in fatty acid compositions under lower temperature conditions were not observed in cultures maintained at 25°C (except 14:0 levels). Both the content and absolute amount of 14:0 gradually increased at 25°C (Table 8). The decrease in 14:0 and 16:0

contents observed at 15°C may be due to the increase in 18:1n-9 and 18:5n-3 contents. A compositional change in fatty acids stimulated under the low temperature was observed for C_{18} fatty acid species: 18:1n-9, 18:3n-6, 18:4n-3 and 18:5n-3 in *E. huxleyi*. According to the fractionation and quantification of each lipid class extracted from *E. huxleyi*, MGDG and phosphatidylcholine (PC) were two major lipid classes in total lipids in *E. huxleyi* (Bell and Pond, 1996). MGDG was dominated by the acyl-chains of C_{18} fatty acid species containing 18:1, 18:3n-3, 18:4n-3 and 18:5n-3 at 70.7% among all fatty acids esterified to the glycerol backbone. PC was also an abundant lipid class and contained only 18:1 at 2.4% of all acyl chains in PC. Thus, synthesis and desaturation of the C_{18} PUFAs may have occurred in the chloroplasts. I then investigated desaturases involved in the synthesis of C_{18} PUFAs in the chloroplasts.

Genomic search for desaturase genes

The whole genome sequence of *E. huxleyi* strain CCMP 1516 has been reported previously (Read et al., 2013). According to the KOG (eukaryotic orthologous groups) annotation by JGI, there are 29 genes encoding proteins similar to the desaturase domain. Eighteen of these genes did not possess the putative Cyt b_5 domain. I then classified the 18 proteins into four groups, Δ9, Δ12, Δ15 and Δ6, based on similarities to the known plastid-type desaturases from cyanobacteria (Table 9). Although Read and co-workers (2013) annotated a Δ15 desaturase (EOD29061), two Δ12 desaturases (EOD07051, EOD26922) and a Δ6 desaturase (EOD40828) based on sequence similarities, while 14 proteins remained unannotated. I further examined the protein, EOD40666, 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 prasinophyte genomes, such as *O. lucimarinus*

(Derelle et al., 2006), *M. pusilla* (Worden et al., 2009) and *B. prasinos* (Moreau et al., 2012). *O. lucimarinus* and *M. pusilla* are also known to produce 18:5n-3 (Ahmann et al., 2011; Dunstan et al., 1992; see Chapter I). 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 11). 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 (Ishida, 2005). For the prediction of these bipartite signal and transit peptides, firstly SignalP (Petersen et al., 2011) was used to estimate the signal peptide and ChloroP (Emanuelsson et al., 1999) 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 (Gruber et al., 2007). Therefore, I 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 11). Because this is relatively short, I estimated

the mature protein started from 75 residues according to the similarity to orthologues.

Since the decrease in culture temperature enhanced the production of 18:1n-9, 18:3n-3 and 18:5n-3 in *E. huxleyi* (Fig 10), accumulation of mRNAs of the desaturases involved in desaturation of these fatty acids would also likely be upregulated. Semi-quantitative PCR clearly showed up-regulation of EhDES15 mRNA at 15°C (Fig 12). Transcriptomic analysis, sequencing and comparison of total cDNA reverse-transcribed from mRNA extracted from 25°C- and 15°C-cultured *E. huxleyi* cells also supported these results. mRNA levels were induced approximately 3.5-fold compared with those from the 25°C conditions (Fig 12; Araie et al., unpublished data). These results indicated that EhDES15 contributed to PUFA production induced under low temperature conditions in *E. huxleyi*. I then performed a functional characterization of EhDES15 using a heterologous expression system.

Heterologous expression of EhDES15 in *Synechocystis* cells

Since EhDES15 possesses a predicted transit-peptide for transportation into plastid and does not contain Cyt b_5 domain, Fe_2-S_2 ferredoxin is likely required as an electron donor for the desaturation reaction. Thus, I used *Synechocystis* cells as a host for heterologous expression. *Synechocystis* is a unicellular photosynthetic microorganism used as a model for photosynthesis studies, since it is highly competent for homologous recombination. This organism contains four desaturases, Δ9, Δ12, Δ6 and Δ15, and produces 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3 and 18:4n-3 (Los and Murata, 2004).

A codon-optimized EhDES15 gene lacking the 74 amino acid-peptide serving as putative bipartite signal and transit peptides at the N-terminus was synthesized and expressed heterologously in *Synechocystis* cells under control of the *trc* promoter (Amann and Brosius, 1985). 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 13A 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 I used in this study (Wada and Murata, 1990) (Fig 13C 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 (Fig 14).

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 (Sakamoto et al., 1994b). I 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 13E 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. I constructed the EhDES15-expressing *Synechocystis* mutant lacking the *desB* gene. The transformant cells accumulated 18:3n-3 and 18:4n-3 (Fig 15).

Discussion

The unicellular haptophyte, *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 (Lang et al., 2011). The 18:5n-3 fatty acid is specifically observed in secondary plants which chloroplasts were originated from red algal symbionys such as haptophytes, dinophytes and raphidophytes, and in only one group of the green microalgae, prasinophytes (Volkman et al., 1998; Ahmann et al., 2011; Dunstan et al., 1992). I first observed changes in PUFA composition at low temperature in *E. huxleyi* (Fig 10). 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 10B). 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*.

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, I 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 *Synechocystis* contains only the ferredoxin-type acyl-lipid desaturases localized in membranes (Los and Murata, 2004) and EhDES15 I expressed was also functional in *Synechocystis* cells as well as native desaturases.

I also found that the Δ15 desaturase was conserved in the genomes of prasinophytes, *O. lucimarinus*, *M. pusilla* and *B. prasinos*, and in a cryptophyta, *Guillardia theta*. In contrast to haptophytes as a secondary plant, prasinophytes are primary plants known as a primitive group of green algae. According to the lipid profiles of *O. lucimarinus* and *M. pusilla*, both synthesize 18:3n-3, similar to *E. huxleyi* (Ahmann et al., 2011; Dunstan et al., 1992; see Chapter I). It is indicated that orthologues of EhDES15 in *O. lucimarinus* and *M. pusilla* catalyse the same Δ15 desaturation reaction to produce 18:3n-3 *in vivo*. Furthermore, both prasinophytes are known to be 18:5n-3 producers. I 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 (2011) identified ER-type Δ4 desaturase which introduces an unsaturated bond at the Δ4 position of 22:5n-3 to produce 22:6n-3 in *O. lucimarinus*. 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 *O. tauri* (van Ooijen et al., 2012), 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 (Fig 16). The tree showed that EhDES15 and orthologues can be classified into a same 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) (Los and Murata, 1994). These results indicate that EhDES15 may be a novel type of Δ15 desaturase.

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 (Sayanova et al., 2011; Pereira et al., 2004), a 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* (Hitz et al., 1994). Domergue and colleagues also used the cells of *Synechococcus* to characterize the substrate specificity of Δ12 plastidial desaturase from a diatom, *P. tricornutum* (Domergue et al., 2003), because *Synechococcus* only produces 18:1n-9 as an unsaturated fatty acid. In this study, I 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 (Wada

and Murata, 1990), and all acyl-lipid desaturases have been characterized (Los and Murata, 2004). The plastidial Δ15 (ω3) desaturase from the sunflower was identified by the gene expression in the model cyanobacterium, *Synechocystis* (Venegas-Calerón et al., 2006). Therefore, using mutant lines lacking each desaturase gene and by controlling the culture temperature, I can obtain any C_{18} substrate *in vivo* to measure substrate specificity of the plastidial desaturase, which can facilitate functional analysis of numerous and divergent plastidial desaturases from non-model photosynthetic organisms. Δ9, Δ12 and Δ6 desaturases in *E. huxleyi* have not been identified, and many desaturase genes have not been identified in the microalgal genome. Heterologous expression of genes in cyanobacteria may allow us to identify and characterize these unknown desaturases.

General Discussion

[Since this paragraph contains an explanation of unpublished data, it is concealed until the paper is published.]

In Chapter II of this Ph.D. thesis, I could observe the induction of 18:5n-3 amount in *E. huxleyi* under low temperature condition (Fig 10). This result suggested the contribution of 18:5n-3 for cold acclimation in *E. huxleyi*. 18:5n-3 is a dominant acyl-chain in galactolipid species in *E. huxleyi* (Bell and Pond, 1996). Furthermore, temperature dependence of the amount 18:5n-3 in glactolipids is also found in another microalga, dinoflagellate, *Borghiella*

dodgei (Flaim et al., 2012). Therefore, regulation of 18:5n-3 level may play an important role to maintain the membrane fluidity of thylakoid membrane, which contributes the tolerance of photosynthetic machinery on thylakoid membrane to low temperature. In order to confirm the actual function of 18:5n-3 in thylakoid membrane, further analysis, e.g. a comparative analysis of photosynthetic activities between *E. huxleyi* and a mutant lacking the activity to synthesize 18:5n-3, is required.

Higher animals including human beings cannot synthesize some PUFAs which are essential and health-beneficial and uptake from our dietary habitat (Riediger et al., 2009). Studies on lipid analysis focusing on PUFAs productivity in photosynthetic organisms has been published from the aspect of applied research for industrial use of PUFAs synthesized. Therefore, production of two PUFAs, 20:5n-3 and 22:6n-3, by various organisms has been well-studied because it is comparably easy to characterize the desaturase involved in 20:5n-3 and 22:6n-3 synthesis: there is an intramolecular Cyt b_5 domain resulting the function when the gene is heterologously expressed in yeast cells. On the other hand, plastid-located acyl-lipid desaturase are only identified in model plants. Using the homology to the primary sequence of a plastid-located desaturase characterized, we can only estimate the genes encoding desaturases in genome of a certain organism. Because of the challenge to establish the transformation approach of the target cells if the genomic sequence became available, there still remain many "predicted" plastid located desaturases encoded in numerous genomes of non-model plants and microalgae. It may be possible to find a peculiar desaturase which is unexpectedly annotated as a typical desaturase, as the Δ15 desaturase, which I found in Chapter II, was formerly thought as Δ6 desaturase by a homology. Taxonomy of photosynthetic eukaryote is quite diverse reflected by the wide variety of shapes, body scales and biochemical features including lipid compositions. To achieve a comprehensive understanding of biosynthesis of unsaturated fatty

acids even in one organism, it requires the functional determination of desaturase one by one, that can be driven by a cyanobacterium as a tool for characterization by heterogenous expression of the plastid-located desaturase.

TABLES AND FIGURES

Table 1. List of microalgae known to produce 18:5.

[Since this table contains an unpublished data, it is concealed until the paper is published.]

Table 2 List of microalgal strain used in Chapter I.

[Since this table contains an unpublished data, it is concealed until the paper is published.]

Table 3. Fatty acid composition of microalgae [fg/cell (mol%/total fatty acid)].

[Since this table contains an unpublished data, it is concealed until the paper is published.]

Table 4. Summary of PUFAs production in green algae.

[Since this table contains an unpublished data, it is concealed until the paper is published.]

Table 5. Genomic search of orthlogous desaturase genes of *Ostreococcus* in the genomes of other microalgae.

[Since this table contains an unpublished data, it is concealed until the paper is published.]

Table 6. 18:5n-3 and DHA contents in haptophycean algae.

Genus Name	Strain Name	18:5n-3 (mol%)	22:6n-3 (mol%)
Emiliania huxleyi (17 strains)	CCMP371	16.4	10.2
	CCMP1516	11.9	21.3
	CCMP2090	14.7	24.3
	NIES-1310	6.8	18.2
	NIES-1311	7.8	5.1
	NIES-1312	17.5	25.7
	NIES-2697	10.2	14.1
	B349	14.9	8.1
	B413	21.0	10.6
	B506	16.4	9.9
	MS-1	13.1	10.8
	RCC1217	4.2	5.5
	MR70N	12.7	8.3
	MR67N	11.5	7.4
	MR65N	7.0	5.5
	MR61N	9.0	7.7
	MR57N	11.6	7.8
Gephyrocapsa oceanica (4 strains)	NIES-353	11.6	10.0
	NIES-838	9.2	14.8
	NIES-1315	17.0	9.9
	NIES-1318	5.5	30.7
Isochrysis galbana (4 strains)	CCMP715	5.7	5.8
	CCMP1323	8.4	5.6
	NIES-2590	0.9	7.5
	UTEX LB 2307	0.9	6.8
Tisochrysis lutea (1 strain)	CCMP463	2.1	8.3
Helladosphaera sp. (1 strain)	Lab. culture	13.4	8.7
Pleurochrysis carterae (1 strain)	Lab. culture	-	5.0

-, not detected.

Table 7. Compositional changes in fatty acids at 15°C in *E. huxleyi*.

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								
16:1n-7	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
18:1n-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
24:1n-9	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								
18:2n-6	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
18:3n-3	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
18:4n-3	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
18:5n-3	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
20:2n-6	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
20:4n-6	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
20:5n-3	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
22:5n-3	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
22:6n-3	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

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

Table 8. Compositional changes in fatty acids at 25°C in *E. huxleyi*.

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								
16:1n-7	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
18:1n-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
24:1n-9	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								
18:2n-6	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
18:3n-3	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
18:4n-3	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
18:5n-3	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
20:2n-6	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
20:4n-6	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
20:5n-3	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
22:5n-3	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
22:6n-3	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

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

Table 9. Predicted proteins involved in unsaturation of fatty acids in *E. huxleyi*.

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

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.

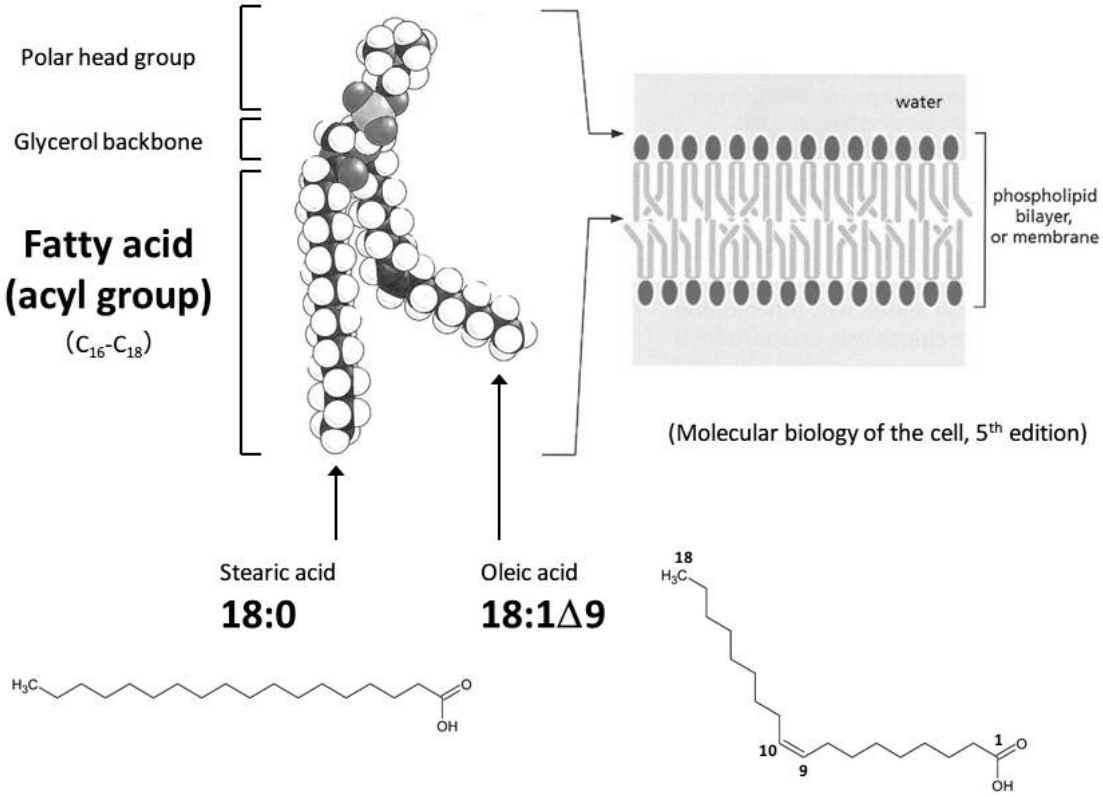


Fig 1. Schematic model of membrane structure and acyl lipid.

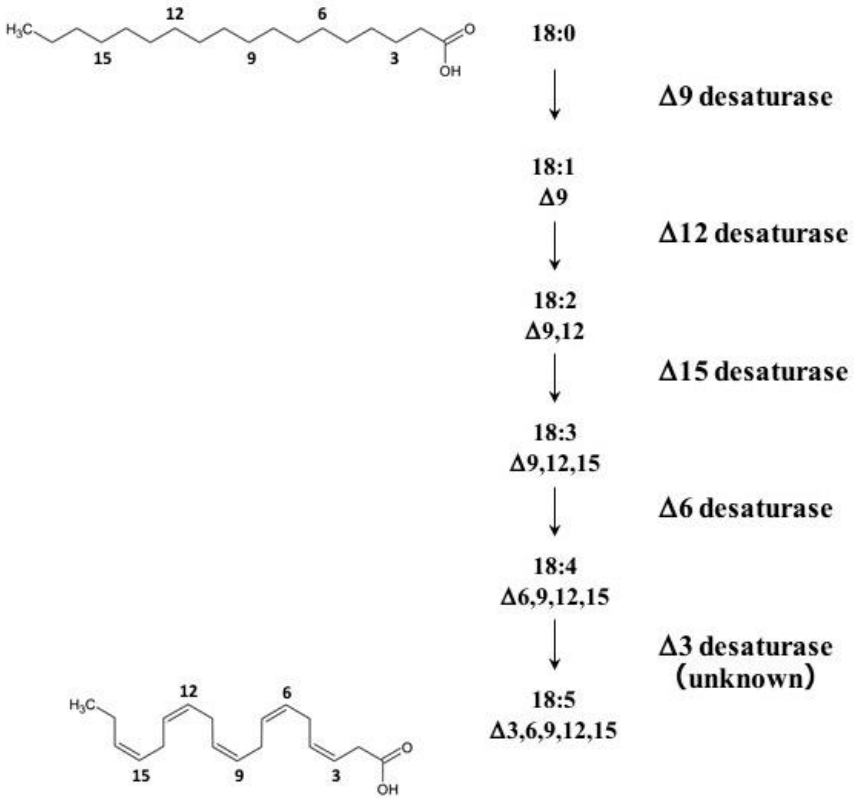


Fig 2. Predicted pathway for 18:5n-3 from 18:0 in *E. huxleyi*.

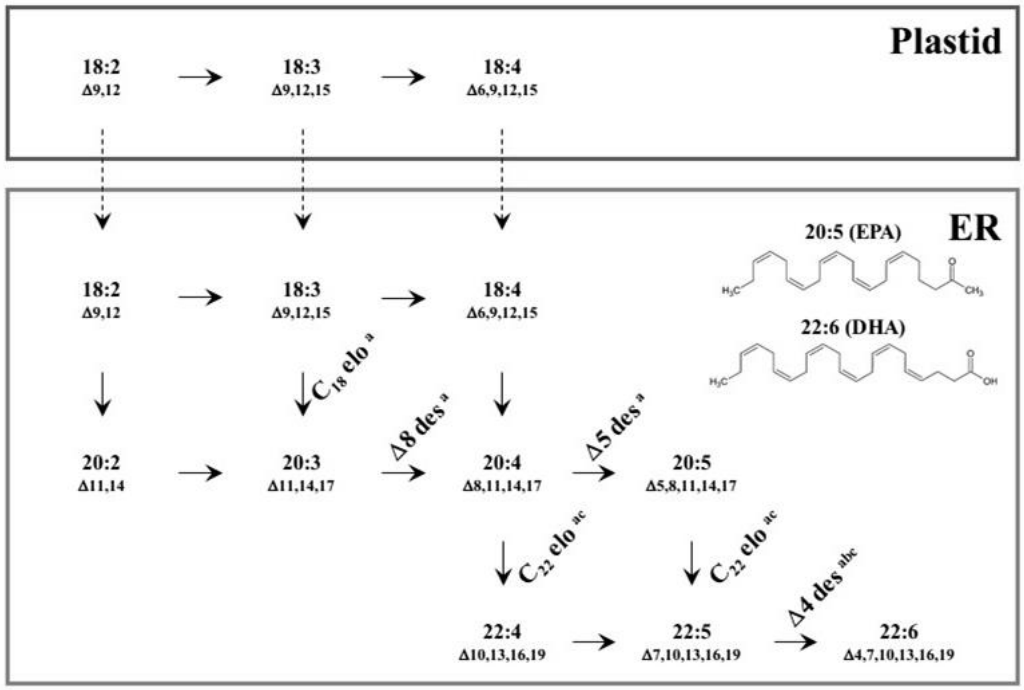


Fig 3. DHA biosynthesis in haptophytes.

[a]*E. huxleyi* (Sayanova et al., 2011), [b]*I. galbana* (Pereira et al., 2004), [c]*P. salina* (Shi et al., 2012)

[Since this figure contains an unpublished data, it is concealed until the paper is published.]

Fig 4. Cell growth of microalgae.

[Since this figure contains an unpublished data, it is concealed until the paper is published.]

Fig 5. Lipid analysis of contaminant in microalgae culture.

[Since this figure contains an unpublished data, it is concealed until the paper is published.]

Fig 6. Composition of major fatty-acids species.

[Since this figure contains an unpublished data, it is concealed until the paper is published.]

Fig 7. Phylogeny and 18:5n-3 production in green plants.

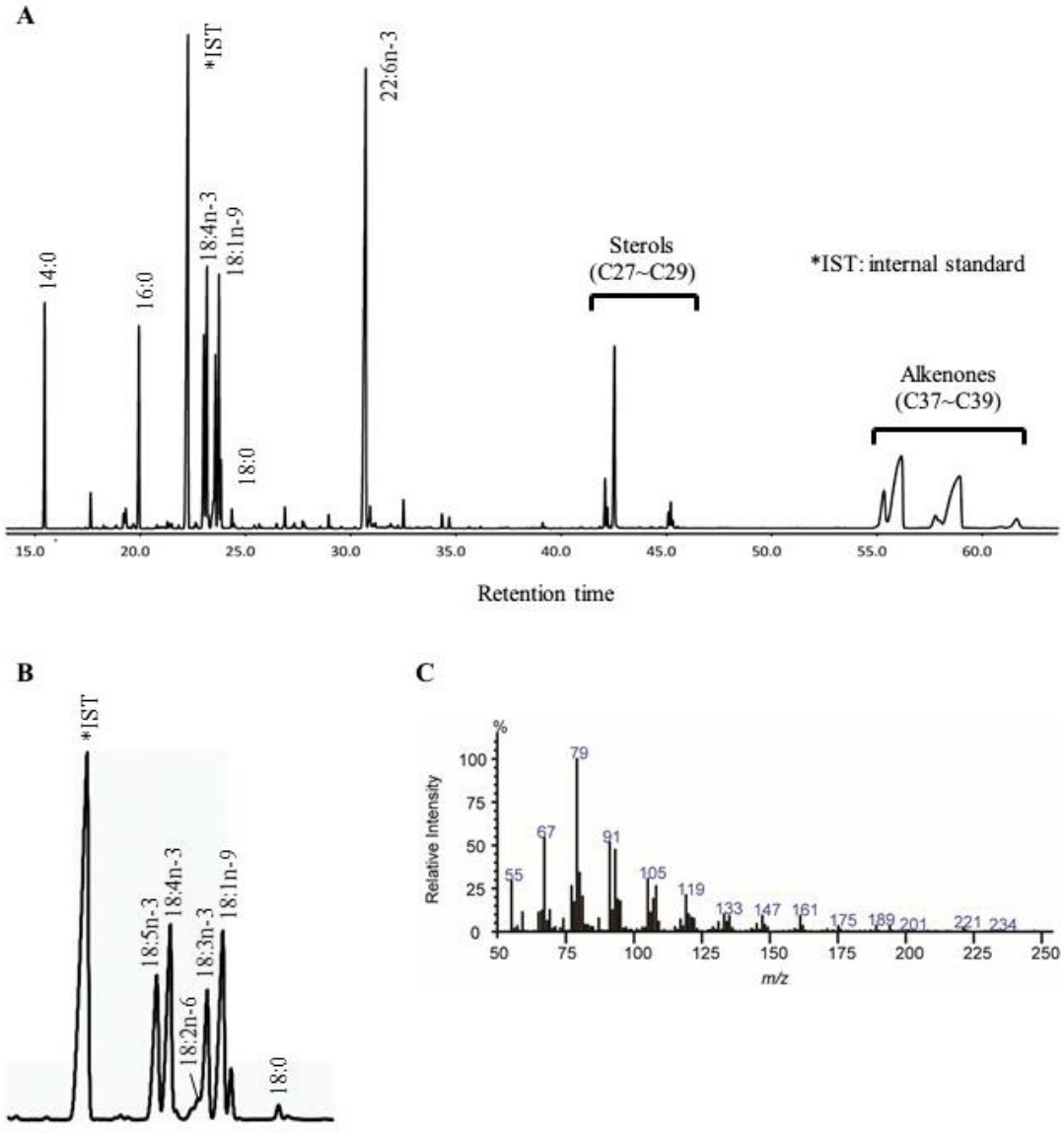
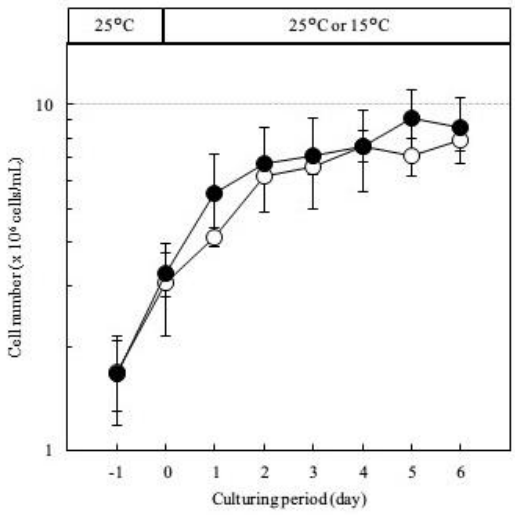


Fig 8. Gas chromatogram of FAMEs extracted from *E. huxleyi*.

A, Methyl-esterified fatty acids of *E. huxleyi* was analysed by GC-FID. Total ion chromatogram was shown (A). IST represents the internal standard of heptadecapentaenoic-acid methyl-ester. B, The chromatogram shows a magnified chromatogram focusing on the peaks for internal standard (*IST, 17:0-methyl ester) and C_{18} derivatives. (B) GC-MS analysis shows the molecular ion peak for 18:5-methyl ester.

A



B

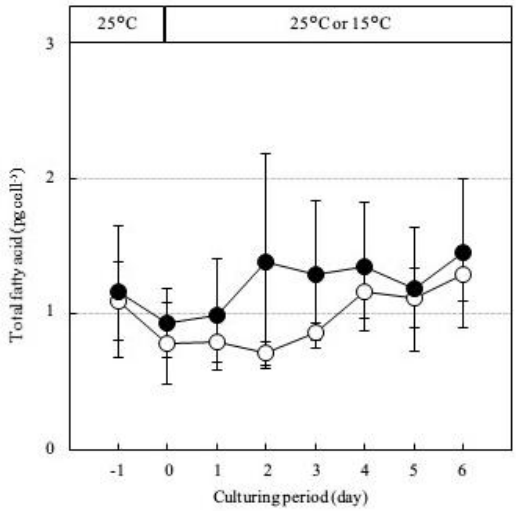
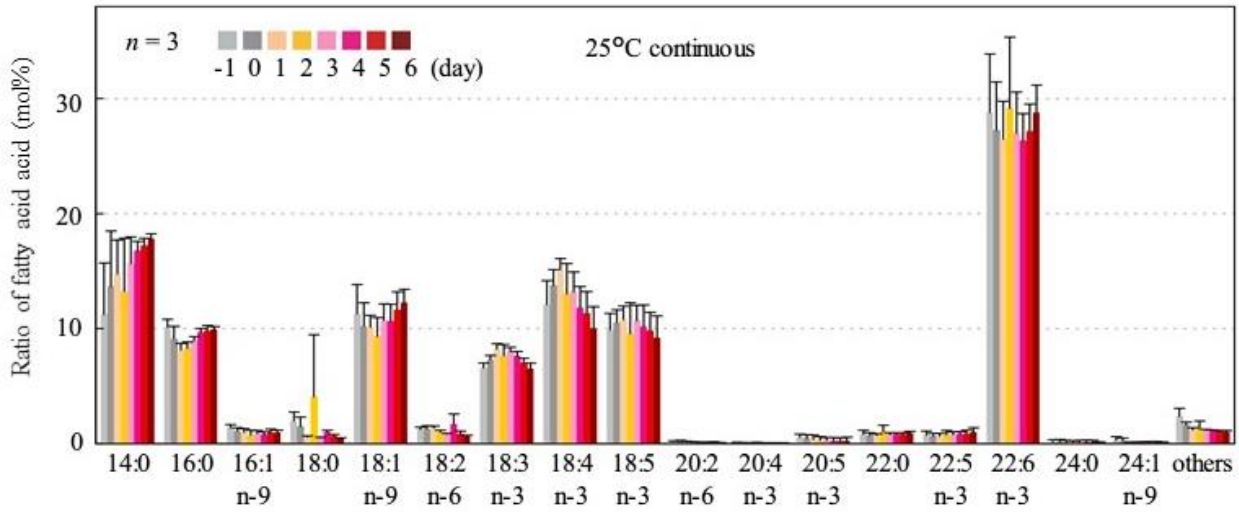


Fig 9. Changes on cell number and total fatty-acids amount of *E. huxleyi* under different temperature condition.

Cell densities (A) and total fatty acids levels (B) are shown by closed circles (25°C) and open circles (15°C). Each point represents the average for three experiments, and the error bars represent the means ± SD.

A



B

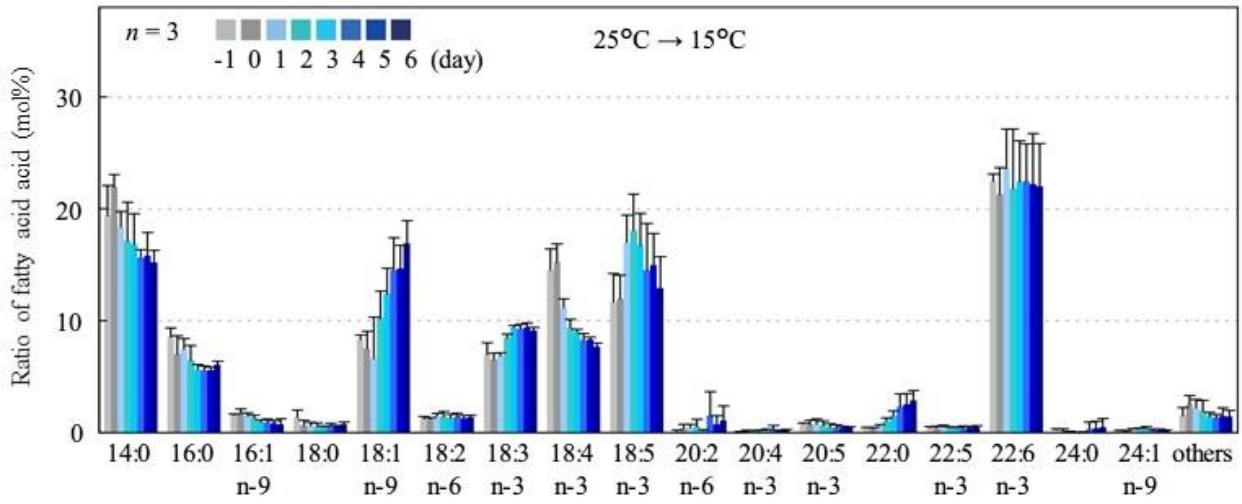


Fig 10. Fatty acid composition of *E. huxleyi* (mol%).

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.

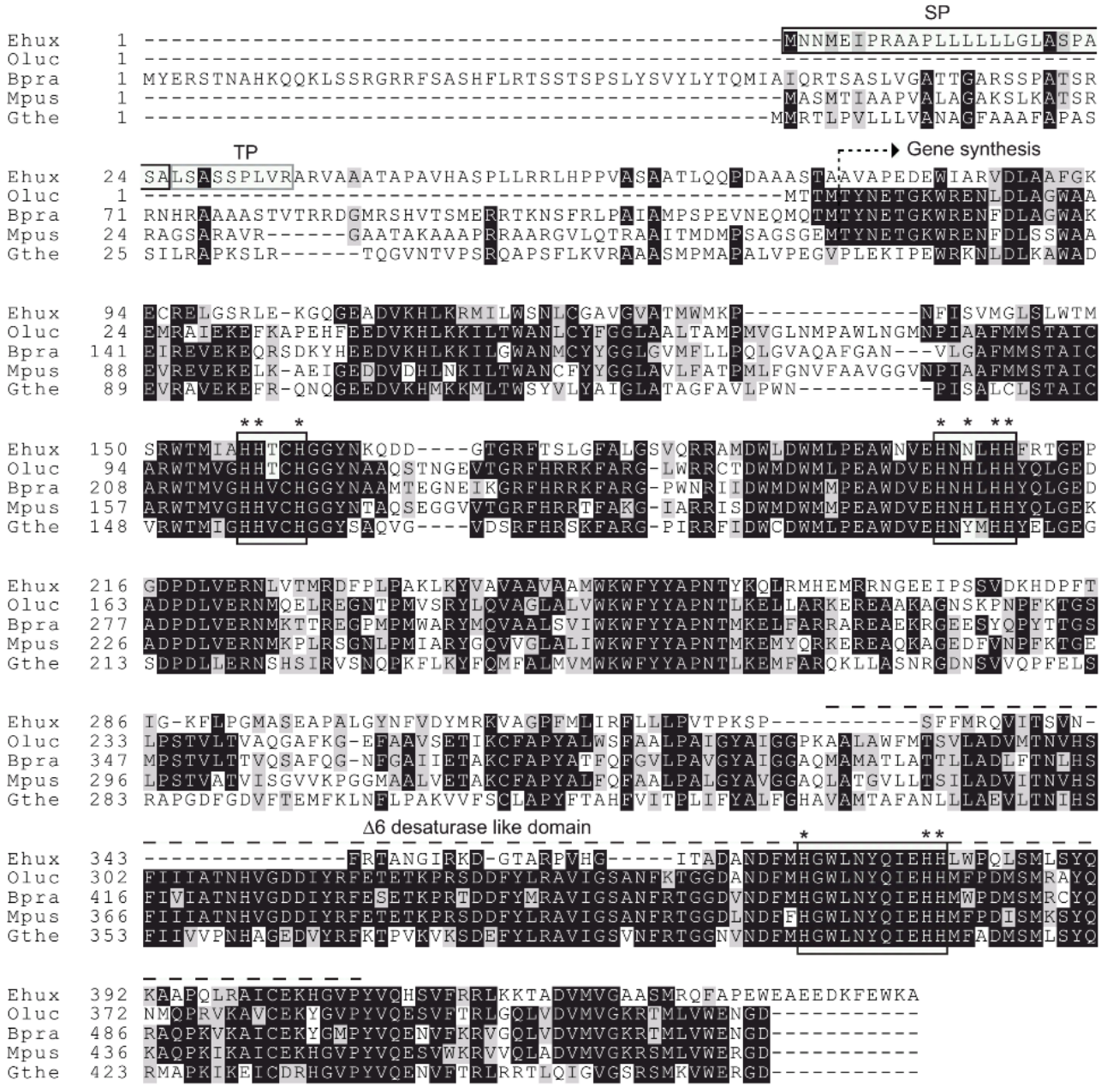

SP
TP
Gene synthesis
Δ6 desaturase like domain
Ehux
Oluc
Bpra
Mpus
Gthe

Fig 11. Multiple alignment of EOD40666 and the orthologous proteins.

Multiple alignment of EhDES15 and orthologous proteins in microalgae. Ehux, *E. huxleyi* EOD40666; Oluc, *O. lucimarinus* (ABO95258); Bpra, *B. prasinos* predicted protein (CCO66881); Mpus, *M. pusilla* CCMP1545 (EEH60489); Gthe, *G. theta* CCMP2712 (EKX48065). The predicted signal peptide (SP) and transit peptide (TP) are enclosed by black and grey rectangles, respectively. Boxes indicate tentative His-boxes, and asterisks indicate histidine residues in the boxes. The region with homology to the Δ6 fatty acid desaturase-like domain is shown by dashed upper-lines. The alignment was drawn by the ClustalW program with full-length amino acid sequences (Thompson et al. 1994).

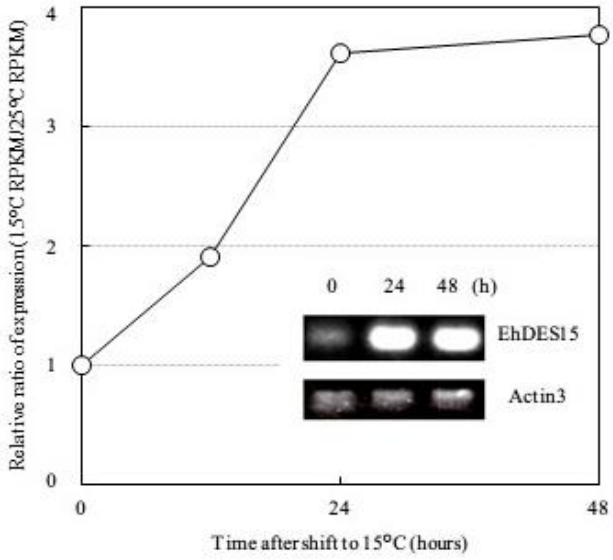


Fig 12. Accumulation of the EhDES15 gene transcript at low temperature.

The relative ratio was calculated based on RPKM (read per kilobase per million mapped reads) values between 15°C and 25°C according to transcriptomic analysis (Araie, unpublished data). RPKM value represents the detection frequency in the transcriptome of a certain gene normalized by its ORF length. The cells were cultured in 25°C and split them into 25°C continuous culturing and 15°C condition. The cells were collected at 0, 12, 24 and 48 h after splitting and total RNAs were extracted. cDNA libraries were sequenced and RPKMs for each coding region of the gene were quantified using the Hiseq 2000 (Illumina, San Diego, CA). The raw RPKM values used for calculation were 54.2, 50.0, 30.8 and 35.4 (0, 12, 24 and 48 h, respectively) in 25°C condition, and 37.7, 66.4, 77.4, 92.8 (0, 12, 24 and 48 h, respectively) in 15°C condition. The results of semi-quantitative PCR analyses of the EhDES15 gene were shown in the inset. The gene for Actin3 was shown as an internal control.

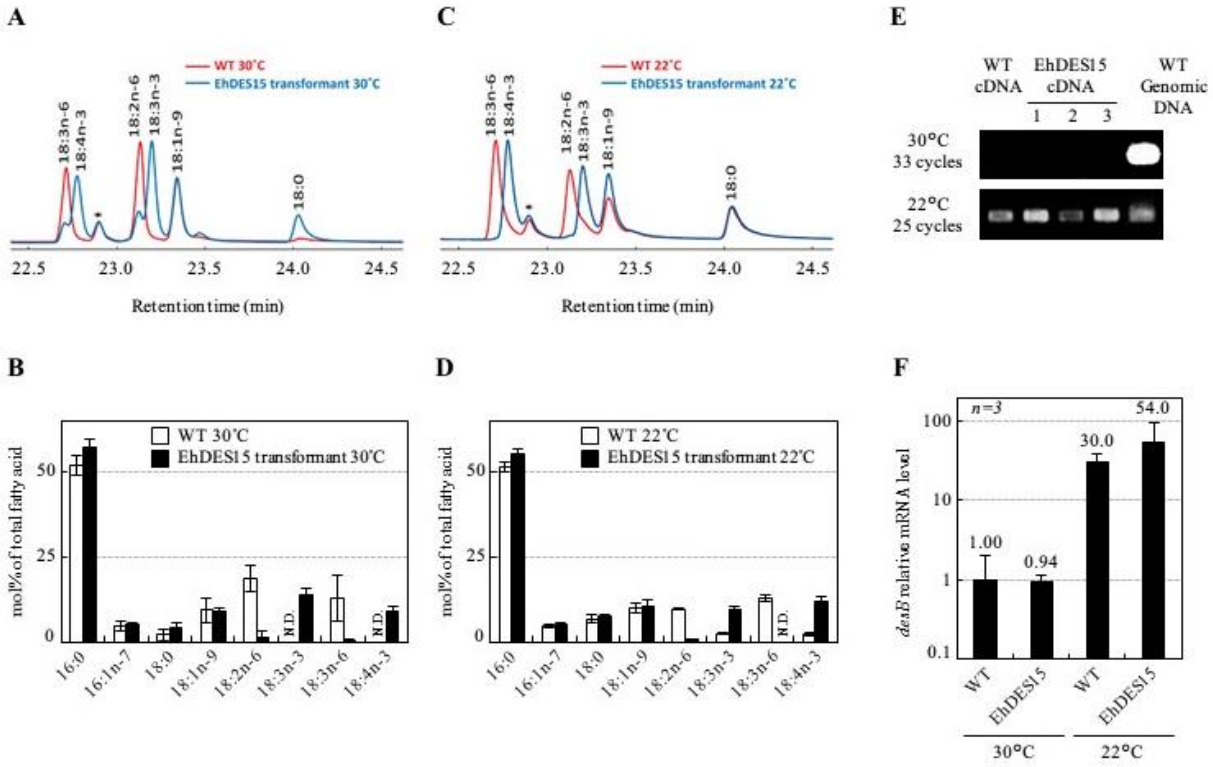


Fig 13. Fatty acid composition in the *Synechocystis* transformant and *desB* mRNA level.

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

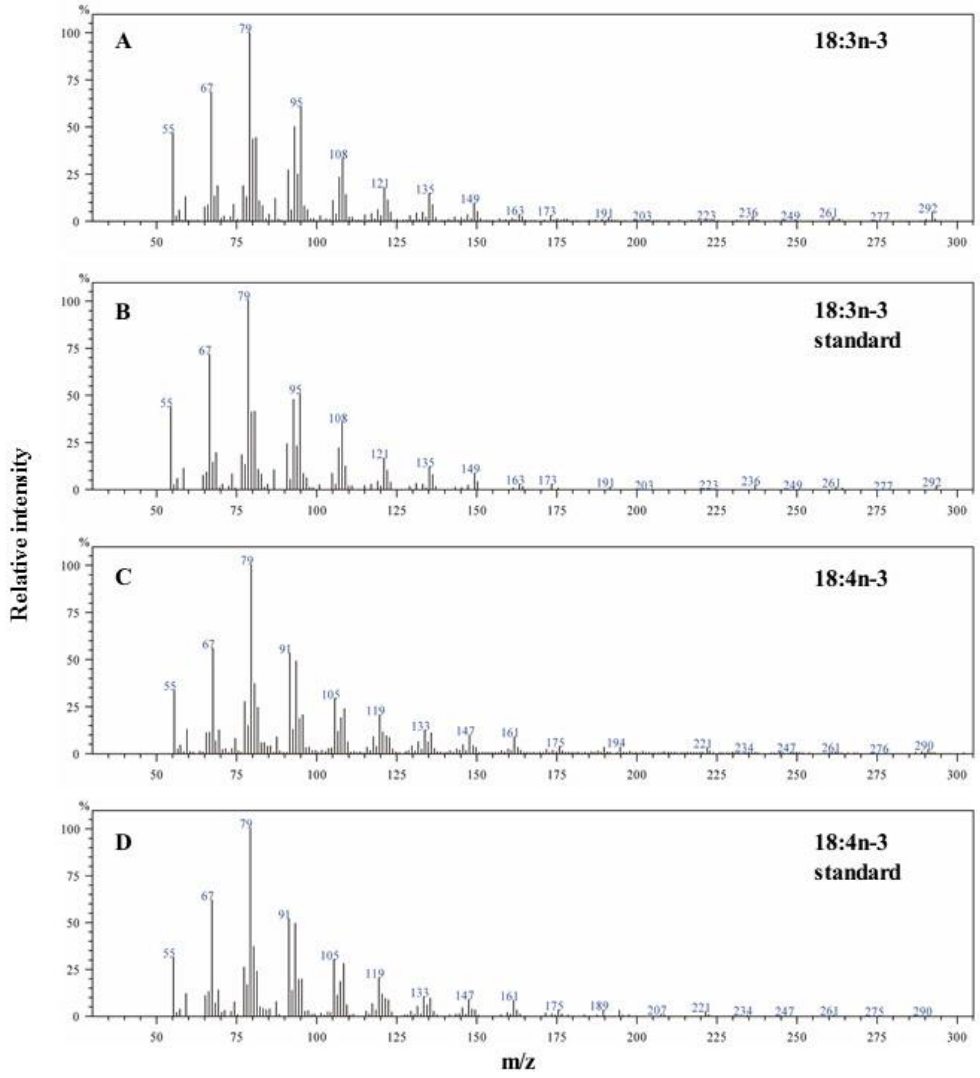


Fig 14. Mass spectrum of 18:3n-3 and 18:4n-3 in *Synechocystis* transformant cells.

Two newly-synthesized peaks were analysed by GC-MS with electron ionization. Each mass spectrum was compared to methyl-esterified 18:3n-3 and 18:4n-3 commercial compounds. A, 18:3n-3 methyl-ester of *Synechocystis* transformant; B, 18:3n-3 methyl-ester standard; C, 18:4n-3 methyl-ester of *Synechocystis* transformant; D, 18:4n-3 methyl-ester standard.

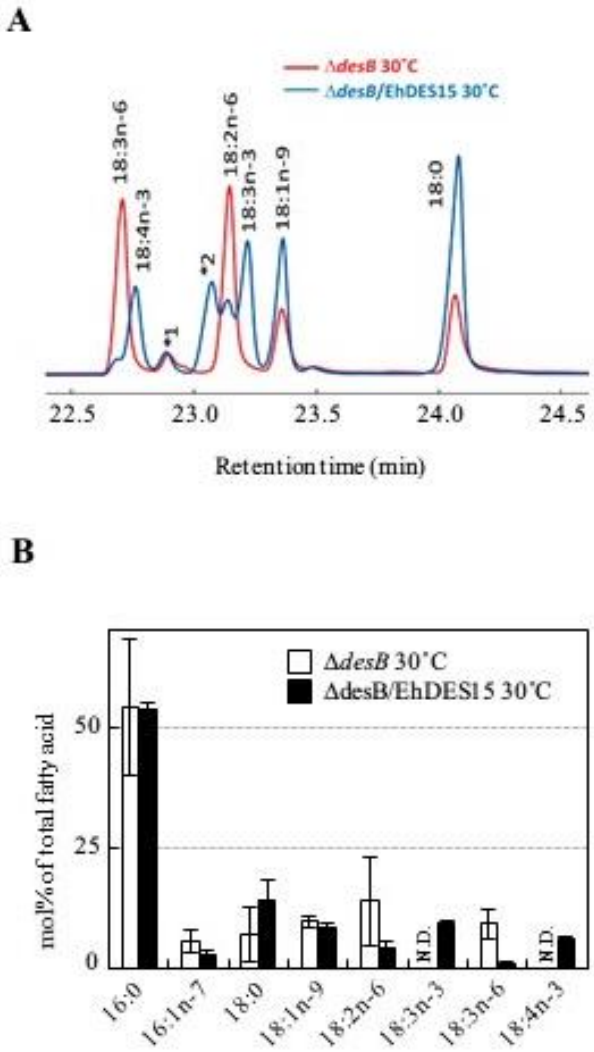


Fig 15. Fatty acid composition of *desB*-disruptant mutant.

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

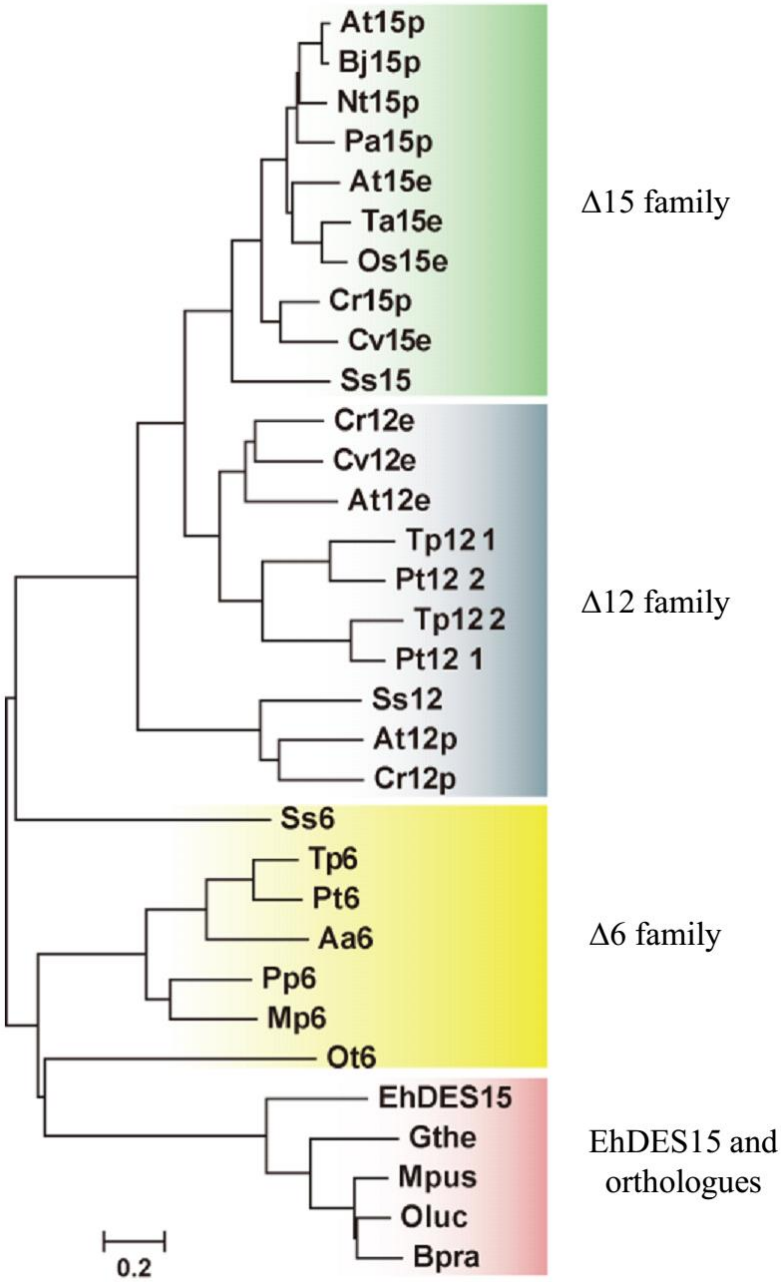

At15p
Bj15p
Nt15p
Pa15p
At15e
Ta15e
Os15e
Cr15p
Cv15e
Ss15
Cr12e
Cv12e
At12e
Tp12 1
Pt12 2
Tp12 2
Pt12 1
Ss12
At12p
Cr12p
Ss6
Tp6
Pt6
Aa6
Pp6
Mp6
Ot6
EhDES15
Gthe
Mpus
Oluc
Bpra
0.2
Δ15 family
Δ12 family
Δ6 family
EhDES15 and orthologues

Fig 16. Unrooted phylogenic tree of the desaturase family.

Tree was drawn by MEGA 6 software (Tamura et al., 2013). Gthe, Mpus, Oluc, Bpra: refer to the legend of Fig 10. Ss6, Ss12 and Ss15 represent *Synechocystis* Δ6 (BAK50679), Δ12 (BAK50342) and Δ15 desaturase (BAK50475), respectively. Pt6, Pt12_1, Pt12_2 represent *P. tricornutum* Δ6 (AAL92563) and two Δ12 desaturases (AAO23565, AAO23564), respectively. Tp6, Tp12_1, Tp12_2: *T. pseudonana* Δ6 (AAX14505) and two Δ12 desaturases (EED90922, EED93612); Aa6: *Aureococcus anophagefferens* Δ6 desaturase (EGB07085); Pp6: *P. patens* Δ6 desaturase (CAA11032); At12p, At12e, At15p, At15e: *A. thaliana* plastid-localized Δ12 (AAL24186), ER-localized Δ12 (AAA32782), plastid-localized Δ15 (AEE75009), ER-localized Δ15 desaturase (AEC08330), respectively; Cr12p, Cr12e, Cr15p: *C. reinhardtii* plastid-localized Δ12 (BAA23881), ER-localized Δ12 (EDP04777), and plastid-localized Δ15 desaturase (EDP09401), respectively; Cv12e, Cv15e: *Chlorella vulgaris* ER-localized Δ12 (BAB78716), Δ15 desaturase (BAB78717); Bj15p: *Brassica juncea* plastid-localized Δ15 desaturase (CAB85467); Nt15p: *Nicotiana tabacum* plastid-localized Δ15 desaturase (BAC01274); Pa15p: *Picea abies* plastid-localized Δ15 desaturase (CAC18722); *Triticum aestivum* ER-localized Δ15 desaturase (BAA28358); Os15e: *Oryza sativa* ER-localized Δ15 desaturase (BAA11397); Mp6: *Marchantia polymorpha* Δ6 desaturase (AAT85663); Ot6: *O. tauri* Δ6 desaturase (AAW70159).

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