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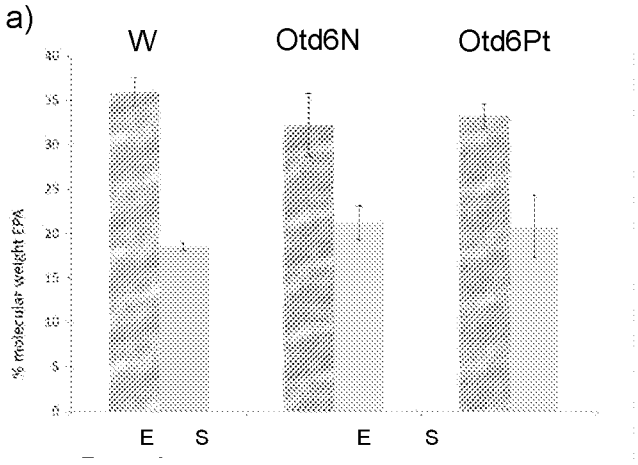

FIGURE 1 20°C 60 µmol photons m^{-2} s^{-}

(57) Abstract: The invention relates to genetically modified organisms with enhanced production of omega-3 long chain polyunsaturated fatty acids.

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Recombinant organisms

Field of the invention

The invention relates to transgenic organisms, in particular transgenic microalgae, with enhanced production of omega-3 long chain polyunsaturated fatty acids, related methods and uses.

Introduction

Long chain polyunsaturated fatty acids (LC-PUFAs) have a carbon backbone of at least 20 carbons in length and contain multiple double-bond desaturations. Long chain polyunsaturated fatty acids can be grouped into either an omega-3 (ϖ–3) or omega-6 (ϖ– 6) category based on the position of the first double bond from the methyl, or ϖ, fatty acid terminus.

It is now well established that omega-3 LC-PUFAs, especially eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ4,7,10,13,16,19) are essential constituents of human nutrition and have key roles in growth and development of infants and children and in maintaining health through their effects on immune system (Voigt et al., 2000; Calder, 2003). There is growing evidence from clinical studies that the presence of omega-3 LC-PUFAs in the human diet has therapeutic effect in conditions such as cardiovascular diseases, obesity, metabolic syndrome and eczema (Navarro et al., 2000; Nugent, 2004; Das, 2002).

Although marine fish is the main dietary source of EPA and DHA, the depletion of fish stocks and pollution of the marine environment indicate an urgent need for an alternative and sustainable source of LC-PUFAs. Marine microorganisms are the primary producers of LC-PUFAs in the aquatic food chain and EPA- and DHA–rich microalgae have been demonstrated to be a promising alternative source to fish oils for human consumption. Thus, commercial cultivation of *Crypthecodinium cohnii and Schizochytrium* sp. have been successfully developed for DHA production and some marine microorganisms have demonstrated potential for the industrial production of EPA (*Nannochloropsis* species*, Phaeodactylum* species*, Nitzshia* spp.) (Harwood and Guschina, 2009). However, commercial production of highly valuable products like

omega-3 LC-PUFAs is expensive to maintain and represents a substantial technological challenge.

One of the approaches to increase the levels of LC-PUFAS is to use acyl-CoA dependent desaturases (Venegas-Caleron et al., 2010). In recent years, considerable focus has been placed on engineering higher plants for the production of very long chain polyunsaturated fatty acids (VLC-PUFAs) in their seed oils. Recently, the advantages of using an acyl-CoA-dependent Δ6-desaturase from *Ostreococcus tauri* (OtD6) to synthesize LC-PUFAs in transgenic Arabidopsis and Camelina plants have been demonstrated (Sayanova O., et al, 2012, Ruiz-Lopez N., et al., 2012). These studies indicate that the first step in the LC-PUFA pathway, the Δ6-desaturation, is rate-limiting.

As an alternative way of producing LC-PUFAs, there is increasing interest in the metabolic engineering of microalgae and genetic modification of algal strains represents a promising strategy to produce sustainable omega-3 oils. Effective recombinant engineering of microalgae to produce increased levels of LC-PUFAs for commercial production would address a global need and microalgae manipulated in this way would be useful as food additives and animal feed, including aquaculture, to meet global demand.

Phaeodactylum tricornutum is an unicellular diatom which accumulates up to 30% EPA and only traces of DHA and is considered a good source for the industrial production of EPA (Molina Grima et al., 1996). The first labelling experiments with [14C]acetate suggested that *P. tricornutum* synthesized EPA de novo by elongation and aerobic desaturation of fatty acids (Moreno et al., 1979). In pulse-chase experiments Arao and Yamada have demonstrated that EPA can be synthesized by 4 different routes and that the preferred route involved intermediates of both omega-6 and omega-3 pathways (Arao and Yamada, 1994). The majority of the EPA was found in galactolipids as opposed to neutral lipids such as triacylglycerol (Arao et al., 1987; Yongmanitchai and Ward, 1993). Recently, the genes encoding the Δ5- and Δ6-desaturases involved in EPA biosynthesis in *P. tricornutum* have been cloned and characterized (Domergue et al., 2002). It was shown that both desaturases were microsomal enzymes contributing equally to both pathways and they supported the preferred route acting simultaneously in omega-6 and omega-3 pathways. This suggests that Δ6- and Δ5- desaturation and

Δ6- elongation involved in biosynthesis of EPA in *P. tricornutum* take place in the endoplasmic reticulum (ER) and newly synthesized EPA is imported after into the plastids. The presence of only minor amounts of all the intermediates of EPA biosynthetic pathway indicates that *P. tricornutum* have developed highly efficient mechanism towards the accumulation of EPA as a single end-product (Arao and Yamada, 1994). In several microalgae DHA can be synthesized by the elongation of EPA to docosapentaenoic acid (DPA; 22:5Δ7,10, 13, 16, 19) by a specific Δ5-elongase, with DPA then converted to DHA by a Δ4-desaturase.

The present invention is aimed at mitigating the shortcomings in the production of LC-PUFAs in various organisms, in particular in algae.

Summary of the invention

The invention generally relates to transgenic organisms, in particular transgenic microalgae, with enhanced production of LC-PUFAs, in particular omega-3 LC-PUFAs such as DHA and/or EPA. The transgenic organisms, in particular transgenic microalgae, express one or more heterologous nucleic acid encoding for a polypeptide involved in the LC-PUFAs biosynthesis pathway. The invention also relates to methods for making transgenic organisms, in particular transgenic microalgae, uses of the transgenic organisms, in particular transgenic microalgae, and methods for increasing the production of LC-PUFAs, in particular omega-3 LC-PUFAs, more particular DHA and/or EPA in an organism, in particular microalgae. The invention also relates to isolated nucleic acids and their uses in methods for the enhanced production of LC-PUFAs, in particular omega-3 LC-PUFAs, in transgenic organisms.

The inventors have shown that microalgae can be manipulated using recombinant methods to produce an increased amount of LC-PUFAs, in particular EPA and DHA using heterologous gene expression. The inventors have surprisingly demonstrated that heterologous expression of Δ5-elongase from *Ostreococcus tauri* alone results in increased accumulation of DHA in *P. tricornutum* with DHA levels in transgenic strains reaching up to 13% of total fatty acids. The inventors have also shown that overexpression of OtD6 in *P. tricornutum* has a positive effect on EPA levels. These findings provide evidence for the efficacy of expressing heterologous genes and enhancing the LC-PUFAs biosynthetic pathway through metabolic engineering in

transgenic microalgae. Furthermore, other organisms that make EPA/DHA, including animals and plants, can be manipulated in the same way by overexpression of Δ5-elongase from *Ostreococcus tauri.*

Accordingly, in one aspect, the invention relates to a transgenic microalgae with increased production of one or more omega-3 LC-PUFA. In one embodiment, the omega-3 LC-PUFA is selected from DHA and/or EPA. In another aspect, the invention relates to the use of a transgenic microalgae in producing omega-3 LC-PUFAs. In another aspect, the invention relates to a method for producing transgenic microalgae with increased omega-3 LC-PUFAs content. In another aspect, the invention relates to a method for increasing production of one of more omega-3 LC-PUFA in microalgae comprising

a) introducing and expressing in a microalgae a heterologous nucleic acid,

b) cultivating said microalgae and

c) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

In another aspect, the invention relates to a method for increasing production of DHA in microalgae. In another aspect, the invention relates to a method for increasing production of EPA in microalgae.

The invention also relates to an oil isolated from a microalgae described herein or a composition comprising a transgenic microalgae described or product therefrom herein and uses thereof.

In another aspect, the invention relates to a method for making a feedstuff comprising
a) cultivating a transgenic microalgae described herein and
b) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

In another aspect, the invention relates to an isolated nucleic acids comprising SEQ ID No. 7 or 9 encoding a Δ6-desaturase (Ost809Δ6) comprising SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 10 and uses thereof. The invention also relates to an isolated nucleic acid comprising SEQ ID No. 15 or 17 encoding a Δ4-desaturase

(Ost809Δ4) comprising SEQ ID No. 16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18 and uses thereof. In another aspect, the invention relates to an isolated nucleic acid comprising SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 and an isolated nucleic acid comprising SEQ ID No. 21 encoding Δ5-desaturase comprising SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22 and uses thereof.

In another aspect, the invention relates to the use of an isolated nucleic described herein in increasing the production of omega-3 LC-PUFAs, in particular DHA and/or EPA, in microalgae or higher plants.

Further, the invention relates to a transgenic organism, preferably a microalgae, with increased DHA levels expressing a heterologous Δ5-elongase.

Figures

The invention is further described in the following non-limiting figures.

Fig. 1. EPA content in WT and transgenic *P. tricornutum* expressing *O.tauri* Δ6 desaturase under different growth conditions at two different growth stages: a) 20°C 60 µmol photons $m^{-2}s^{-1}$; b) 20°C 25 µmol photons $m^{-2}s^{-1}$; c) 18°C 25 µmol photons $m^{-2}s^{-1}$

Fig. 2a. Total fatty acid composition of WT and transgenic *P. tricornutum* cells expressing OtElo5 during the exponential (E) and stationary (S) phases. Cultures were grown at 20°C under constant illumination 60 µmol photons. m^{-2} s^{-1} with agitation. Each value represents the mean ± SD of 3 separate experiments.

b. EPA< DPA and DHA content in WT and transgenic *P. tricornutum* expressing OtElo5. Cultures were grown at 20°C 60 µmol m-2s-1 under constant agitation at 70 rpm. Each measurement is the average of 3 biological replicates.

Fig. 3. The acyl-CoA profiles of WT (A) and transgenic P. tricornutum expressing the Ostreococcus Elo5 (B). The accumulation of LC-PUFA acyl-CoAs in B is boxed with a dotted line.The internal standard (istd) is 17:00 acyl-CoA.

Fig. 4. EPA and DHA content in the total FA extracts of WT and transgenic OtElo5 *P. tricornutum* cells.

Fig. 5A. The distribution of TAG species from WT and transgenic *P. tricornutum* at stationary phase of growth.

Fig. 5B. The distribution of TAG species from WT and transgenic *P. tricornutum* at different stages of growth.

Fig. 6. The distribution of DHA in TAG species from WT and transgenic *P.tricornutum* expressing OtElo5 at different stages of the growth cycle: A-DHA in specific TAGs; B-% of TAG containing DHA.

Fig. 7. Omega-3 PUFA biosynthetic pathway (schematic representation).

Fig. 8. Expression of Ost809Δ6-desaturase in transgenic yeast in the presence of the exogenous substrate 18:3n-3 (ALA). (BPX72 column). Note the conversion of ALA to the higher unsaturated form (SDA - arrowed). No conversion occurs with yeast strains containing the empty vector (pYES2 - C), and only when the expression of the Ost809 desaturase is induced by the addition of galactose (Gal +; B)

Fig. 9. Functional characterization of Ost809Δ6 in yeast (BPX72 column). Yeast cells supplemented with LA and ALA. Expression of *Ostreococcus 809* Δ6 in yeast, supplemented with both 18:2 (LA) and 18:3 (ALA). Note the specific conversion of ALA, but not LA, to a higher unsaturated. No conversion occurs with yeast strains containing the empty vector (pYES2 - C), and only when the expression of the Ost809 desaturase is induced by the addition of galactose (Gal +; B)

Fig. 10. FAMEs profile of transgenic yeast expressing Ost809Δ4 desaturase in the presence of DPA (C22:5n-3). *Expression of Ostreococcus 809* Δ4 in yeast cells supplemented with exogenous 22:5 (DPA). Note the conversion of 22:5n-3 to the higher unsaturated form (22:6n-3; DHA - arrowed). No conversion occurs with yeast strains containing the empty vector (pYES2 - C), and only when the expression of the Ost809 D4 desaturase is induced by the addition of galactose (Gal +; B). NB. These C22 PUFAs are best resolved on a HP1 GC column – in this case, the (poly)unsaturated fatty acids eluted earlier than less saturated forms – this is the inverse compared to BPX72 column used above

Fig. 11. FAMEs profile of transgenic yeast expressing FcElo6 (BPX72 column). Yeast were supplemented with 18:3n-6 (GLA). *Expression of Fragilariopsis cylindrus* Elo6 in yeast cells supplemented with exogenous 18:3 (GLA). Note the conversion of 18:3 ALA to the elongated form 20:3n-3 (arrowed). No conversion occurs with yeast strains

containing the empty vector (pYES2 - C), and only when the expression of the Fragilariopsis Elo6 is induced by the addition of galactose (Gal +; B).

Fig. 12. Phylogenetic tree showing relationship between n-3 specific Ost809Δ6 desaturase and other Δ6-desaturases.

Fig. 13. Expression of FcElo6 resulted in increase of DHA levels up to 14-17%. GC-MS analysis of total FA profiles from Pt cells expressing FcElo6.

Fig. 14. Schematic representation of vector system pPTOS2.

Figure 15. Co-expression of two heterologous omega-3 LC-PUFA biosynthetic activities in P. tricornutum. Fatty acid composition of Pt_WT, pPhOS2.1 (expressing OtElo5) and pPhOS2.2 (expressing OtD6Pt and OtElo5) cells during the S phase of growth at 16°C and 20°C. Values are the average of three experiments (+/- standard error).

Fig. 16. Fatty acid composition of pPhOS_Ppglut (expressing OtElo5 and Ppglucose transporter) cells during the S phase of growth at 20°C, 100 µmol $m^{-2}s^{-1}$ under constant agitation at 70 rpm. N=1.

Fig. 17. Fatty acid composition of pPhOS_Hsglut (expressing OtElo5 and human glucose transporter) cells during the S phase of growth at 20°C, 100 µmol $m^{-2}s^{-1}$ under constant agitation at 70 rpm. N=1.

Fig. 18. Growth of Wt and pPhOS_Ppglut Pt cells in the dark.

Detailed description

The present invention will now be further described. In the following passages, different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature.

The invention relates to the genetic manipulation of the fatty acid biosynthetic pathway in microalgae. In particular, the invention relates to methods for increasing the

production of LC-PUFAs, in particular omega-3 LC-PUFAs, for example one of more omega-3 LC-PUFA in an organism, in particular in microalgae.

Polyunsaturated fatty acids can be classified into two major families, depending on the position (n) of the first double bond nearest the methyl end of the fatty acid carbon chain. Thus, the omega-6 fatty acids (ϖ–6) have the first unsaturated double bond six carbon atoms from the omega (methyl) end of the molecule and additionally have a total or two or more double bonds, with each subsequent unsaturation occurring 3 additional carbon atoms toward the carboxyl end of the molecule. In contrast, the omega-3 fatty acids (ϖ–3) have the first unsaturated double bond three carbon atoms away from the omega end of the molecule and additionally have a total of three or more double bonds with each subsequent unsaturation occurring 3 additional carbon atoms towards the carboxyl end of the molecule.

Table I summarizes the common names of omega-3 fatty acids and the abbreviations that will be used throughout the specification:

Table I

Common Name	Abbreviation	Shorthand notation
oleic acid	OA	$18:1^{\Delta 9}$
Linoleic acid	LA	$18:2^{\Delta 9,12}$
γ-Linolenic acid	GLA	$18:3^{\Delta 6,9,12}$
di-homo γ-linolenic acid	DGLA	$20:3^{\Delta 8,11,14}$
Arachidonic acid	ARA	$20:4^{\Delta 5,8,11,14}$
α- linolenic acid	ALA	$18:3^{\Delta 9,12,15}$
stearidonic acid	SDA	$18:4^{\Delta 6,9,12,15}$
eicosatetraenoic acid	ETA	$20:4^{\Delta 8,11,14,17}$
eicosapentaenoic acid	EPA	$20:5^{\Delta 5,8,11,14,17}$
docosapentaenoic acid	DPA	$22:5^{\Delta 7,10,13,16,19}$
docosahexaenoic acid	DHA	$22:6^{\Delta 4,7,10,13,16,19}$

There are a number of enzymes that are involved in the omega-3 PUFA biosynthetic pathway as shown in figure 7. These include desaturases and elongases.

A variety of genes involved in oil production have been identified through genetic means in different organisms and the DNA sequences of some of these genes are publicly available. Non-limiting examples are shown below:

Accession No.	Description
AY131238	*Argania spinosa* Δ6-desaturase
Y055118	*Echium pitardii var. pitardii* Δ6-desaturase
AY055117	*Echium gentianoides* Δ6-desaturase
AF296076	*Mucor rouxii* Δ6-desaturase
AF007561	*Borago officinalis* Δ6-desaturase
L11421	*Synechocystis sp* Δ6-desaturase
NM_031344	*Rattus norvegicus* Δ6 fatty acid desaturase
AF465283,	*Moritierella alpine* Δ6 fatty acid desaturase
AF465282	*Moritierella isabellina* Δ6 fatty acid desaturase
AF419296	*Pythium irregulare* Δ6 fatty acid desaturase
AB052086	*Mucor circinelloides* D6d mRNA for Δ6 fatty acid desaturase
AJ250735	*Ceratodon purpureus* mRNA for Δ6 fatty acid desaturase
AF126799	*Homo sapiens* Δ6 fatty acid desaturase
AF126798	*Mus musculus* Δ6 fatty acid desaturase
AF199596,	*Homo sapiens* Δ5 desaturase
AF320509	*Rattus norvegicus* liver Δ5 desaturase
AB072976	*Mus musculus* D5D mRNA for Δ5 desaturase
AF489588	*Thraustochytrium sp.* ATCC21685 Δ5 desaturase
AJ510244	*Phytophthora megasperma* mRNA for Δ5 fatty acid desaturase
AF419297	*Pythium irregulare* Δ5 fatty acid desaturase
AF07879	*Caenorhabditis elegans* Δ5 fatty acid desaturase
AF067654	*Mortierella alpina* Δ5 fatty acid desaturase
AB022097	*Dictyostelium discloideum* mRNA for Δ5 fatty acid desaturase
AF489589.1	*Thraustochytrium sp.* ATcc21685 Δ4 fatty acid desaturase
AY332747	*Pavlova lutheri* Δ4 fatty acid desaturase (des1) mRNA
AAG36933	*Emericella nidulans* oleate Δ12 desaturase

AF110509,	*Mortierella alpina* Δ12 fatty acid desaturase mRNA
AAL13300	*Mortierella alpina* Δ12 fatty acid desaturase mRNA
AF417244	*Mortierella alpine* ATCC 16266 Δ12 fatty acid desaturase
AF161219	*Mucor rouxii* Δ12 desaturase mRNA
X86736 S	*Piruline platensis* Δ12 desaturase
AF240777	*Caenorhabdtitis elegans* Δ12 desaturase
AB007640	*Chlamydomonas reinhardtii* Δ12 desaturase
AB075526	Chorella vulgaris Δ12 desaturase
AP002063	*Arabidopsis thaliana* microsomal Δ12 desaturase
NP_441622,	*Synechocystis sp.* PCC6803 Δ15 desaturase
AAL36934	*Perilla frutescens* Δ15 desaturase

All references to sequence IDs herein are specifically incorporated by reference.

Additionally, the patent literature provides many additional DNA sequences of genes (and/or details concerning several of the genes above and their methods of isolation) involved in polyunsaturated fatty acid production (see, for example: U.S. Pat. No. 5,968,809 (Δ5-desaturases); U.S. Pat No. 5,972,664 and U.S. Pat. No. 6,075,183 (Δ5 desaturases); WO 91/13972 and U.S. Pat. No. 5,057,419 (Δ9-desaturases); WO 93/11245 (Δ15-desaturases); WO 94/11516. U.S. Pat. No. 5,443,974 and WO 03/099216 (Δ12-desaturases); U.S. 2003/0196217 A1 (Δ17-desaturase); WO 02/090493 (Δ4-desaturases); and WO 00/12720 and U.S. 2002/0139974A1 (elongases)).

The term “desaturases” as used herein refers to a polypeptide component of a multi-enzyme complex that can desaturate, i.e. introduce a double bond in one or more fatty acids to produce a mono- or polyunsaturated fatty acid or precursor of interest. Some desaturates have activity on two or more substrates. It may be desirable to empirically determine the specificity of a fatty acid desaturase by transforming a suitable host with the gene for the fatty acid desaturase and determining its effect on the fatty acid profile of the host. Nucleic acids that encode for desaturases are isolated from various organisms can be used according to the various aspects of the invention and examples are described herein, including *Ostreococcus sp.*

Desaturases include omega-3-desaturase, Δ6-desaturase, Δ5-desaturase, Δ12-desaturase, Δ19-desaturase, Δ17-desaturase and Δ4-desaturase.

The term "elongase" as used herein refers to a polypeptide that can elongate a fatty acid carbon chain to produce an acid two carbons longer than the fatty acid substrate that the elongase acts upon. Nucleic acids that encode for elongases isolated from various organisms can be used according to the various aspects of the invention and examples are described herein, including *Ostreococcus sp.*

Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, SDA to ETA, ARA to DTA and EPA to DPA. In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree and type of unsaturation.

For example, a C14/16 elongase will utilize a C14 substrate (e.g., myristic acid), a C16/18 elongase will utilize a C16 substrate (e.g., palmitate), a C18/20 elongase will utilize a C18 substrate (e.g., GLA, SDA, LA, ALA) and a C20/22 elongase (also referred to as a Δ5-elongase) will utilize a C20 substrate (e.g., ARA, EPA).

Since some elongases have broad specificity, a single enzyme may be capable of catalyzing several elongase reactions (e.g., thereby acting as both a C16/18 elongase and C18/20 elongase). It may be desirable to empirically determine the specificity of a fatty acid elongase by transforming a suitable host with the gene for the fatty acid elongase and determining its effect on the fatty acid profile of the host.

Elongases include Δ6-, Δ5- and Δ9-elongases. Δ5-elongase is not generally viewed as rate limiting in the production of DHA and it is generally assumed that the first step in the LC-PUFA pathway, the D6-saturation, is rate-limiting.

Embodiments of the invention relating to the production of omega-3 LC-PUFAs in transgenic microalgae are described below. A skilled person would understand that these embodiments are not limited to transgenic microalgae, but can be applied to other organisms to produce omega-3 LC-PUFAs. The organism may be an animal, for example a mammal. In one embodiment, humans are specifically excluded. In another embodiment, the organism is a plant, for example a crop plant.

In a first aspect, the invention relates to a transgenic microalgae with increased production of omega-3 LC-PUFAs, for example one or more omega-3 LC-PUFA or total omega-3 LC-PUFA content. According to the various aspects of the invention, the omega-3 LC-PUFAs may be selected from SDA, ETA, EPA, DPA or DHA. In one embodiment, the omega-3 LC-PUFAs is DHA. In another embodiment, the omega-3 fatty acid is EPA.

According to the various aspects of the invention described herein, the increase in the production of DHA or EPA is measured as an individual content of different omega-3 LC-PUFAs in total fatty acids (TFA). In other words, the increase is measured as a percentage of the total fatty acid content. Preferably, the increase is at least 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% or more compared to a control microalgae (mol %).

In one embodiment, the omega-3 LC-PUFAs is DHA. In the transgenic microalgae of the invention, the DHA content is increased by at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% or more compared to a control microalgae. In one embodiment, the omega-3 LC-PUFAs is DHA. In the transgenic microalgae of the invention, the DHA content is at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10 fold higher than in a control microalgae. Preferably, the total DHA content is at least 10% of the total fatty acid content (mol %).

In another embodiment, the omega-3 LC-PUFAs is EPA. In the transgenic microalgae according to the various aspects of the invention, the EPA content is increased by at least 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%. Preferably, the total EPA content is at least 20% of the total fatty acid content (mol %).

According to the various aspects of the invention, the total fatty acid content, LC-PUFAs content, omega-3 LC-PUFAs content or the content of individual fatty acids such as DHA is increased compared to a control microalgae. A control microalgae as used herein is a microalgae which has not been modified according to the methods of the invention. Accordingly, the control microalgae has not been genetically modified to express a nucleic acid as described herein to alter LC-PUFA content. In one embodiment, the control microalgae is a wild type microalgae. In another embodiment,

the control microalgae is a microalgae that does not carry a transgene according to the methods described herein, but expresses a different transgene. The control microalgae is typically of the same algae species.

The term "total fatty acids content" herein refers to the sum of all cellular fatty acids that can be derivitized to fatty acid methyl esters by the base transesterification method in a given sample (known as the art, for example as described in Sayanova et al., (1997); Sayanova et al., (2003) FEBS Lett. 2003 May 8;542(1-3):100-4).

According to the various aspects of the invention, the increase is measured in the stationary phase.

According to the various aspects of the invention, the term microalgae encompasses all microalgae which have the capacity to make LC-PUFAs. The algae may be a heterotrophic or autothrophic algae.

A skilled person would know that the term "microalgae" includes unicellular, photosynthetic microorganisms from several distinct biological groups, comprising, for example, eukaryotic chlorophyta, rhodophyta, heterokont, haptophyta divisions of algae and prokaryotic cyanobacteria.

EPA has been found in a wide variety of marine microalgae including in the classes *Bacillariophyceae* (diatoms), *Chlorophyceae*, *Chrysophyceae, Cryptophyceae*, *Eustigamatophyceae* and *Prasinophyceae* (see Table II). Accordingly, according to the various aspects of the invention, the microalgae may be selected from these orders, classes or species.

According to the various aspects of the invention, the microalgae may be selected from a microalgae listed in Table II.

Table II: Proportions of PUFAs in marine microalgae
**Emiliania huxleyi* is the now accepted name for *Coccolithus huxleyi*

Omega-3 LC-PUFAs (% of Total Fatty acids)			
Mircoalgae sp. (Order/class/sp.)	**EPA**	**DHA**	**References**
Chlorophyta (green algae)			
Chlorophyceae			
Chlorella minutissima	45.0	-	Seto et al., (1984)
Prasinophyceae			
Ostreococcus tauri	2.0	12.0	Wagner M. et al., (2010)
Ostreococcus lucimarinus	2.1	3.8	Ahmann et al., (2011)
Hetermastrix rotundra	28	7	Yongmanitchai and Ward, (1989)
Haptophyta			
Pavlovophyceae			
Pavlova lutheri	11.6	9.1	Tonon et al., (2002)
Prymnesiophyceae			
Isochrysis galbana	22.6	8.4	Molina Grima et al., (1995)
Emilinaia huxleyi *	17	-	Yongmanitchai and Ward, (1989)
Cryptophyceae			
Cryptomonadaceae			
Cryptomonas maculate	17	-	Yongmanitchai and Ward, (1989)
Chromonas sp.	12	6.6	Renaud et al., (1999)
Cryptomonas sp.	16	10	Yongmanitchai and Ward, (1989)
Rhodomonas sp.	8.7	4.6	Renaud et al., (1999)
Heterokont			
Bacillariophyceae (diatoms)			

Asterionella japonica	20	-	Yongmanitchai and Ward, (1989)
Amphora coffeaformis	1.39	0.39	Renaud et al., (1999)
Biddulphia sinensis	24.0	1.0	Yongmanitchai and Ward, (1989)
Chaetoceros sp.	16.7	0.8	Renaud et al., (1999)
Cylindrotheca fusiformis	18.8	-	Tan and Johns, (1996)
Fragilaria pinnata	6.8	1.0	Renaud et al., (1999)
Nitzchia angularis	21	-	Kyle et al., (1992)
Navicula incerta	25.2	-	Tan and Johns, (1996)
Navicula pelliculosa	9.4	-	Tan and Johns, (1996)
Navicula saprophila	16.0	-	Kitano et al., (1997)
Nitzschia closterium	15.2	-	Renaud et al., (1994)
Nitzschia frustulum	23.1	-	Renaud et al., (1994)
Nitzschia laevis	19.1	-	Wen and Chen, (2001)
Phaeodactylum tricornutum	34.5	-	Yongmanitchai and Ward, (1991)
Skeletonema costatum	29.2	3.4	Blanchemain and Grizeau, (1999)
Thalassiosira pseudonana	12.2	-	Tonon et al., (2002)
Chrysophyceae (golden algae)			
Monochrysis lutheri	19	-	Yongmanitchai and Ward, (1989); Kyle, (1992)
Pseudopedinella sp.	27	-	Yongmanitchai and Ward, (1989)
Crisosphaera carterae	20	-	Yongmanitchai and Ward, (1989)
C.elongate	28	-	Yongmanitchai and Ward, (1989)
Eustigmatophyceae			
Nannochloropsis salina	15	-	Yongmanitchai and Ward, (1989)
Nannochloropsis sp.	35	-	Sukenik, (1991)
Nannochloris sp.	27	-	Yongmanitchai and Ward, (1989)
Monodus subterraneus	32.9	-	Quiang et al., (1997)

In one embodiment, autotrophic microalgae which are as the primary producers of PUFAs are preferred. For example, the microalgae may be selected from *Phaeodactylum, Nannochloropsis, Thraustochytrium* or *Schizochytrium.* Other genera

include *Spirulina*, *Dunaliella*, *Chlorella*, *Thalassiosira*, *Isochrysis, Porphyridium, Nannochloropsis, Pavlova, Chaetoceros, Crypthecodinium, Fraigilariopsi* and *Nitzshia.*

For example, the microalgae may be selected from *Chaetoceros calcitrans, Isochrysis galbana, Pavlova lutheri, Pseudoisochrysis paradoxa, Tetraselmis suecica* and *Skeletonema costatum, Nannochloropsis oculata, Thalassiosira pseudonana, Pavlova lutheria, Porphyridium irregular, Crypthecodinium cohnii, Porphyridium purpureum* and *Porphyridium cruentum.*

In one embodiment, the microalgae is a diatom. Diatoms are brown algae found throughout marine and freshwater ecosystems that are responsible for around 20% of global primary productivity. A defining feature of diatoms is their ornately patterned silicified cell wall (known as frustule), which display species-specific nanoscale-structures.

The diatom may be a centric diatoms or a pennate diatom. In one embodiment, the diatom belongs to the order of Naviculales. In one embodiment, the diatom is *P. tricornutum* or *Thalassiosira pseudonana*. In a preferred embodiment, the diatom is *P. tricornutum.* In another embodiment, the diatom is *Fragilariopsis sp.* for example *Fragilariopsis cylindrus.*

A skilled person would understand that the aspects of the invention are not limited to *P. tricornutum*. Indeed, a skilled person would understand that the invention can be applied to any microalgae that has the capacity to synthesise EPA and/or DHA.

The transgenic microalgae according to the various aspects of the invention expresses one or more heterologous transgenes which encode for one or more nucleic acid involved in the biosynthesis of LC-PUFAs. “Heterologous" with respect to sequence means a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. The heterologous transgene is preferably derived or isolated from a microalgae. In one embodiment, the heterologous transgene is derived or isolated from *Prasinophyceae*, for example *Ostreococcus sp*. Sequences of heterologous transgenes may be modified to be codon optimised for expression in the target organism. Thus, the invention relates to transgenic organisms obtained through recombinant methods.

For example, the heterologous transgene may encode for one or more of a Δ15-desaturase, a Δ6-desaturase, a Δ5-desaturase, a Δ4-desaturase, a Δ12-desaturase, a Δ5-elongase, Δ6-elongase or combinations thereof.

In one embodiment, the transgenic microalgae expresses a heterologous nucleic acid encoding a Δ5-elongase. Thus, in one aspect, the invention relates to a transgenic microalgae expressing a nucleic acid encoding a Δ5-elongase. For example, the transgenic microalgae expresses a nucleic acid encoding a Δ5-elongase, but does not express any other transgene encoding for a polypeptide involved in the regulation of the LC-PUFAs biosynthetic pathway. In other embodiments, the transgenic microalgae expresses a nucleic acid encoding a Δ5-elongase and one or more additional heterologous transgene involved in the regulation of the LC-PUFAs biosynthetic pathway, for example a Δ6-desaturase such as OtD6 as shown in example 4. Thus, embodiments where nucleic acids encoding a Δ5-elongase and a Δ6-desaturase are co-expressed are specifically part of the invention. Δ5-elongases and Δ6-desaturases are as defined herein.

In one embodiment, the transgenic microalgae described herein co-expresses a heterologous nucleic acid which is not involved in the regulation of the LC-PUFAs biosynthetic pathway, for example a glucose transporter gene as shown in example 5 together with a heterologous nucleic acid involved in the regulation of the LC-PUFAs biosynthetic pathway such as OtElo5. As shown in the example, a vector can be used allowing co-expression of two heterologous nucleic acids involved in the regulation of different traits - one for omega-3s, and one which allows the alga to be grown in the dark, by the expression of a glucose transporter. If the cells are then provided with an exogenous carbon source such as glucose, they can grow in the dark. Thus, in one embodiment, an exogenous carbon source such as glucose is provided when culturing algae expressing a gene involved in the regulation of the LC-PUFAs biosynthetic pathway such as OtElo5 and a glucose reporter. Examples of nucleic acids that can be used according to the invention encoding a glucose reporter are shown in SEQ ID No. 23 and SEQ ID No. 25. Respective peptides are shown in SEQ ID No. 24 and SEQ ID No. 26.

As used herein, the words "nucleic acid", "nucleic acid sequence", "nucleotide", or "polynucleotide" are intended to include DNA molecules (e.g. cDNA or genomic DNA), RNA molecules (e.g., mRNA), natural occurring, mutated, synthetic DNA or RNA molecules, and analogs of the DNA or RNA generated using nucleotide analogs. It can be single-stranded or double-stranded. Such nucleic acids or polynucleotides include, but are not limited to, coding sequences of structural genes, anti-sense sequences, and non-coding regulatory sequences that do not encode mRNAs or protein products. These terms also encompass a gene. The term "gene" or “gene sequence“ is used broadly to refer to a DNA nucleic acid associated with a biological function. Thus, genes may include introns and exons as in genomic sequence, or may comprise only a coding sequence as in cDNAs, and/or may include cDNAs in combination with regulatory sequences. In one embodiment of the various aspects of the invention, cDNA sequences synthetic (deduced) open reading frames, analogous to cDNA are preferred.

For the purposes of the invention, "transgenic", “transgene” or "recombinant" means with regard to, for example, a nucleic acid sequence, an expression cassette, gene construct, a vector or an autonomous replicating element such as an artificial chromosome comprising the nucleic acid sequence or an organism transformed with the nucleic acid sequences, expression cassettes or vectors according to the invention, all those constructions brought about by recombinant methods in which either

(a) the nucleic acid sequences encoding proteins useful in the methods of the invention, or

(b) genetic control sequence(s) which is operably linked with the nucleic acid sequence according to the invention, for example a promoter, or

(c) a) and b)

are not located in their natural genetic environment or have been modified by recombinant methods, such as mutagenesis, it being possible for the modification to take the form of, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The natural genetic environment is understood as meaning the natural genomic or chromosomal locus in the original microalgae or the presence in a genomic library.

A transgenic microalgae for the purposes of the invention is thus understood as meaning a microalgae which comprises within its nuclear and or plastidial genome a

heterologous polynucleotide. The heterologous polynucleotide is preferably stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct.

In the context of the present invention, a Δ5-elongase catalyzes the conversion of EPA to DPA. Thus, any nucleic acid that encodes a Δ5-elongase that catalyzes the conversion of EPA to DPA may be used according to the various aspects of the invention as a transgene. In one embodiment, the Δ5-elongase used in the present invention is derived or isolated from *Ostreococcus*, preferably *Ostreococcus tauri*. Preferably, the Δ5-elongase is OtElo5 derived or isolated from *Ostreococcus tauri*. In one embodiment, the transgenic microalgae according to the invention expresses a nucleic acid comprising SEQ ID No. 1, a functional variant thereof or a sequence that encodes for a Δ5-elongase wherein said elongase has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 2. In a preferred embodiment, the microalgae is *P. triconutum* and the nucleic acid encodes a Δ5-elongase comprising or consisting of SEQ ID No. 2.

A functional variant as used according to the aspects of the invention is a biologically active variant. For example, a biologically active variant of SEQ ID No. 1 is a nucleic acid sequence, which, when expressed in a microalgae such as *P. tricornutum*, increases production of DHA. The term variant includes sequences which have been altered for codon optimisation for expression in the target organism for example for expression in *P. tricornutum*.

Thus, it is understood, as those skilled in the art will appreciate, that the aspects of the invention, which use certain polynucleotides including the methods and uses, encompasses more than the sequence specified, but also include alterations in the peptide that do not affect the biological function. For example, alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue,

such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

In one embodiment, the said nucleic acid according to the various aspects of the invention is operably linked to a regulatory sequence.

The terms "regulatory element" is used interchangeably herein with "control sequence" and "promoter" and all terms are to be taken in a broad context to refer to regulatory nucleic acid sequences capable of effecting expression of the sequences to which they are ligated. The term "promoter" typically refers to a nucleic acid control sequence located upstream from the transcriptional start of a gene and which is involved in recognising and binding of RNA polymerase and other proteins, thereby directing transcription of an operably linked nucleic acid. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue- specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or -10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative that confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Suitable promoters are identified in the examples. For example, if the microalgae is *P. tricornutum*, the promoter may be the *P. tricornutum* promoter fcpA. However, a skilled person would understand that other promoters can also be used. For example, suitable

promoters may also be selected from inducible promoters which respond to specific environmental or chemical stimuli.

The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

The transgene may be part of a vector which, in addition to one or more regulatory sequences also comprises selection markers. These are known in the art. Transformation of microalgae may be carried out by standard procedures known in the art, for example by particle bombardment or electroporation.

The transgenic microalgae expressing a nucleic acid encoding a Δ5-elongase is characterised by an increase in DHA and DPA compared to a control microalgae. In particular, the increase, as measured as a percentage of the total fatty acid content is at least 2, at least 3, at least 4, at least 5, at least 6, at least, at least 8, at least 9 or at least 10 fold higher than in a control microalgae. Specifically, the DHA content is at least 2, at least 3, at least 4, at least 5, at least 6, at least, at least 8, at least 9 or at least 10 fold higher than in a control microalgae. Preferably, the total DHA content is at least 10% of the total LC-PUFAs content (%mol). In one embodiment, the transgenic microalgae expressing a nucleic acid encoding a Δ5-elongase does not express a second transgene encoding for another polypeptide involved in the regulation of the LC-PUFAs pathway, preferably in the regulation of the omega-3 LC-PUFAs pathway.

In one embodiment of the various aspects of the invention, the transgenic microalgae expressing a heterologous nucleic acid encoding a Δ5-elongase may further express one or more additional heterologous nucleic acid encoding for one or more polypeptide involved in the regulation of the LC-PUFAs pathway, preferably in the regulation of the omega-3 LC-PUFAs pathway. In other words, the transgenic microalgae comprises one or more further transgene encoding for one or more polypeptide involved in the regulation of the LC-PUFAs pathway. The polypeptide is preferably selected from any desaturase or elongase involved in the omega-3 PUFA biosynthetic pathway as shown in figure 7. Any combination of desaturase and elongase may also be used. Thus, the nucleic acid may encode for one or more of a Δ15-desaturase, a Δ6-desaturase, a Δ5-

desaturase, a Δ4-desaturase, a Δ6-desaturase, a Δ5-elongase, Δ6-elongase or combinations thereof.

In one embodiment, the nucleic acid encodes a Δ6-desaturase. In the context of the present invention, a Δ6-desaturase catalyzes the conversion of ALA to SDA and also LA to GLA. Δ6-Desaturases are described in WO 93/06712, US 5,614, 393, US 5614393, WO 96/21022, WO 02/1557 and WO 99/27111 and their application to production in transgenic organisms is also described, e.g. in WO 98/46763, WO 98/46764 and WO 98/46765. In one embodiment, the Δ6-desaturase used in the present invention is derived or isolated from *Ostreococcus*, preferably OtD6 from *Ostreococcus tauri* (Domergue et al (2005), AY746357). In one embodiment, the nucleic acid comprises SEQ ID No. 3 or 5 and encodes a 6Δ-desaturase comprising or consisting of SEQ ID No. 4 or 6, a functional variant thereof or a polypeptide that encodes for a 6Δ-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 4 or 6.

In another embodiment, the Δ6-desaturase is from the microalgae *Ostreococcus* RCC 809. Preferably, the nucleic acid comprises SEQ ID No. 7 or 9 and encodes a 6Δ-desaturase from the microalgae *Ostreococcus* RCC 809 comprising or consisting of SEQ ID No. 8 or 10, a functional variant thereof or a sequence that encodes for a 6Δ-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 8 or 10.

In another embodiment, the nucleic acid encodes for a Δ4-desaturase. According to the various aspects of the invention, a Δ4-desaturase may be derived or isolated from *E. huxleyi*. Thus, in one embodiment, the nucleic acid comprises SEQ ID No. 11 encoding a Δ4-desaturase comprising or consisting of SEQ ID No. 12, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 12.

In another embodiment, the Δ4-desaturase is derived or isolated from *T. pseudonana*. Thus, in one embodiment, the nucleic acid comprises SEQ ID No. 13 encoding a Δ4-desaturase comprising or consisting of SEQ ID No. 14, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 14.

In another embodiment, the Δ4-desaturase is derived or isolated from *Ostreococcus* RCC809. In one embodiment, the nucleic acid comprises SEQ ID No. 15 or 17 encoding a Δ4-desaturase comprising or consisting of SEQ ID No. 16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 16 or 18.

In another embodiment, a Δ6-elongase is from *Fragilariopsis cylindrus.* In one embodiment, the nucleic acid comprises SEQ ID No 19 encoding a Δ6-elongase comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 20.

In another embodiment, a Δ5-desaturase is from *Fragilariopsis cylindrus.* In one embodiment, the nucleic acid comprises SEQ ID No 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 22.

In another aspect, the transgenic microalgae of the invention expresses a heterologous nucleic acid encoding a Δ6-desaturase, a Δ5-desaturase, a Δ4-desaturase, Δ6-elongase or combinations thereof. These enzymes are defined herein.

In one aspect, a transgenic microalgae of the invention expresses a heterologous nucleic acid encoding a Δ6-desaturase. Thus, in another aspect, the invention also relates to transgenic microalgae expressing a heterologous nucleic acid encoding a Δ6-desaturase. For example, the transgenic microalgae expresses a nucleic acid encoding a Δ6-desaturase, but does not express any other transgene involved in the regulation of the LC-PUFAs biosynthetic pathway. In other embodiments, the transgenic microalgae expresses a Δ6-desaturase and additional transgenes involved in the regulation of the LC-PUFAs biosynthetic pathway, for example a Δ5-elongase such as OtElo5 as shown in the examples.

In one embodiment, the microalgae is *P. triconutum*. In one embodiment, the nucleic acid comprising or consisting of SEQ ID No. 3 or 5 encodes a Δ6-desaturase or a sequence that encodes for a Δ6-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 4 or 6. In a preferred embodiment, the microalgae is *P. triconutum* and the nucleic acid encodes a Δ6-desaturase comprising or consisting of SEQ ID No. 4 or 6.

The transgenic microalgae expressing a nucleic acid encoding a Δ6-desaturase is characterised in that the total fatty acids content, specifically the omega 3 LC-PUFA content, is altered compared to a control microalgae. In particular, the omega-3 LC-PUFA content is increased by at least 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% or more. Specifically, the EPA content is increased by at least 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% compared to a control microalgae. Preferably, the total EPA content is at least 20% of the total LC-PUFAs content (mol %). Moreover, the DHA content in the transgenic algae is also increased by at least 0.5%.

In one embodiment, the various aspects of the invention exclude embodiments that relate to the production of biofuels.

In another aspect, the invention relates to a method for producing transgenic microalgae with increased omega-3 LC-PUFA content comprising introducing and expressing in a microalgae a heterologous nucleic acid which encodes for a polypeptide involved in the LC-PUFAs biosynthetic pathway. The omega-3 fatty acid may be selected from ALA, SDA, ETA, EPA, DPA or DHA. In one embodiment, the omega-3 LC-PUFAs is DHA. In another embodiment, the omega-3 fatty acid is EPA. The nucleic acid may encode Δ6-desaturase, Δ5-desaturase, Δ4-desaturase, Δ5-elongase, Δ6-elongase or combinations thereof.

In one embodiment, the method relates to producing transgenic microalgae with increased DHA levels said method comprising transforming a microalgae with a heterologous nucleic acid encoding a Δ5-elongase. According to this embodiment, the method may further comprise transforming said microalgae with one or more additional heterologous nucleic acid that regulates the production of omega-3 fatty acids, for example transforming with a nucleic acid encoding a Δ6-desaturase. In another embodiment, no additional nucleic acid that regulates the production of omega-3 fatty acids is introduced into said microalgae and expressed as heterologous nucleic acids.

In another embodiment, the invention relates to a method for producing transgenic microalgae with increased EPA levels said method comprising transforming a microalgae with a nucleic acid encoding a Δ6-desaturase. According to this embodiment, the method may further comprise transforming said microalgae with one or more additional nucleic acid that regulates the production of omega-3 LC-PUFAs. In another embodiment, no additional nucleic acid that regulates the production of omega-3 fatty acids is introduced into said microalgae.

In one embodiment, the method comprises transforming said microalgae with one or more additional nucleic acid that does not regulates the production of omega-3 LC-PUFAs, for example a glucose transporter gene.

Microalgae obtained or obtainable by those methods are also within the scope of the invention.

In another aspect, the invention relates to a method for increasing production of one of more omega-3 LC-PUFA in microalgae comprising

a) cultivating a transgenic microalgae described herein and

b) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

Specifically, the invention relates to a method for increasing the production of one or more omega-3 LC-PUFAs in microalgae comprising:

a) introducing and expressing in a microalgae a heterologous nucleic acid which encodes for a polypeptide involved in the LC-PUFAs biosynthetic pathway,

b) cultivating a transgenic microalgae expressing said heterologous nucleic acid and

c) obtaining one or more omega -3 fatty acid from the transgenic microalgae.

The transgenic microalgae is as described herein and is cultivated under conditions which allow for the production of one or more omega-3 LC-PUFAs. The nucleic acid may encode a Δ15-desaturase, a Δ6-desaturase, a Δ5-desaturase, a Δ4-desaturase, a Δ12-desaturase, Δ5-elongase, Δ6-elongase or combinations thereof as described herein.

In one embodiment, the method relates to increasing DHA production in microalgae comprising

a) introducing and expressing in a microalgae a heterologous nucleic acid encoding a Δ5-elongase,

b) cultivating a transgenic microalgae expressing said heterologous nucleic acid and

c) obtaining DHA from the transgenic microalgae.

The microalgae as described herein. The Δ5-elongase is as described herein. In one embodiment, the microalgae does not include and express a second heterologous nucleic acid encoding an enzyme involved in the regulation of the synthesis of omega-3 LC-PUFAs. In another embodiment, the microalgae includes and expresses a second heterologous nucleic acid encoding a polypeptide involved in the regulation of the synthesis of omega-3 LC-PUFAs. In another embodiment, the microalgae includes and expresses a second heterologous nucleic acid encoding a polypeptide not involved in the regulation of the synthesis of omega-3 LC-PUFAs, for example a glucose

transporter. The transgenic microalgae is cultivated under conditions which allow for the production of DHA.

In one embodiment, the method relates to increasing DHA production in microalgae comprising

a) introducing and expressing in *P. triconutum* a heterologous nucleic acid encoding a Δ5-elongase,

b) cultivating *P. triconutum* expressing said heterologous nucleic acid and

c) obtaining said DHA from *P. triconutum*.

The microalgae as described herein. The Δ5-elongase is as described herein. In one embodiment, the microalgae does not include and express a second heterologous nucleic acid encoding an enzyme involved in the regulation of the synthesis of omega-3 LC-PUFAs. In another embodiment, the microalgae includes and expresses a second heterologous nucleic acid encoding an enzyme involved in the regulation of the synthesis of omega-3 LC-PUFAs. In another embodiment, the microalgae includes and expresses a second heterologous nucleic acid encoding a polypeptide not involved in the regulation of the synthesis of omega-3 LC-PUFAs, for example a glucose transporter.

P. triconutum is cultivated under conditions which allow for the production of DHA. These conditions will be apparent to the skilled person. For example, preferred culture conditions for *P. triconutum* are about 20°C under constant illumination in about 60-80 μmol photons m- $^{2}s^{-1}$. In one embodiment, the method comprises transforming said microalgae with one or more additional nucleic acid that does not regulates the production of omega-3 LC-PUFAs, for example a glucose transporter gene and supplying an exogenous carbon source. The algae can be grown in the dark.

In another embodiment, the method relates to increasing EPA in microalgae comprising:

a) introducing and expressing in a microalgae a heterologous nucleic acid encoding a 6Δ-desaturase,

b) cultivating the transgenic microalgae and

c) obtaining said EPA from the transgenic microalgae.

The microalgae as described herein The 6Δ-desaturase is as described herein. The microalgaeis cultivated under conditions which allow for the production of EPA.

In one embodiment, the method relates to increasing EPA production in microalgae comprising

a) introducing and expressing in *P. triconutum* a heterologous nucleic acid encoding a 6Δ-desaturase,
b) cultivating *P. triconutum* and
c) obtaining said EPA from *P. triconutum*.

The microalgae as described herein The Δ6-desaturase is as described herein. *P. triconutum* is cultivated under conditions which allow for the production of EPA.

These conditions will be apparent to the skilled person. For example, preferred culture conditions for *P. triconutum* are about 20°C under constant illumination in about 0-80μmol photons m- $^{2}s^{-1}$ or preferably about 18°C under constant illumination in about 25μmol photons m- $^{2}s^{-1}$. In one embodiment, the method comprises transforming said microalgae with one or more additional nucleic acid that does not regulates the production of omega-3 LC-PUFAs, for example a glucose transporter gene and supplying an exogenous carbon source. The algae can be grown in the dark.

In another aspect, the invention relates to a method for the manufacture of an oil, lipid or fatty acid composition comprising
a) cultivating a transgenic microalgae as described herein under conditions which allow for the production one or more omega-3 LC-PUFAs and
b) obtaining said one or more omega-3 LC-PUFAs from the transgenic microalgae.

In preferred embodiment, the omega-3 LC-PUFAs is DHA or EPA.

In another aspect, the invention relates to an omega-3 LC-PUFAs or oil isolated from a transgenic microalgae as described herein.

The fatty acids produced by the processes of the present invention can be isolated from the microalgae in the form of an oil, a lipid or a free fatty acid. One embodiment of the invention is therefore oils, lipids or fatty acids or fractions thereof which have been

produced by the methods of the invention, especially preferably oil, lipid or a fatty acid composition comprising EPA or DHA and being derived from the transgenic microalgae.

The term "oil", or "lipid" is understood as meaning a fatty acid mixture comprising unsaturated, preferably esterified, fatty acid(s). The oil or lipid is preferably high in omega-3 polyunsaturated or, advantageously, esterfied fatty acid(s). In a particularly preferred embodiment the oil or lipid has a high ALA, ETA, EPA, DPA and/or DHA content, preferably a high EPA and/or DHA content.

For the analysis, the fatty acid content can, for example, be determined by gas chromatography after converting the fatty acids into the methyl esters by transesterification of the lipids such as triacylglycerides and/or phospholipids.

The omega-3 polyunsaturated acids produced in the method of the present invention, for example EPA and DHA, may be in the form of fatty acid derivatives, for example sphingolipids, phosphoglycerides, lipids, glycolipids, phospholipids, monoacylglycerol, diacylgycerol, triacylgylcerol or other fatty acid esters.

The omega-3 and other polyunsaturated fatty acids which are present can be liberated for example via treatment with alkali, for example aqueous KOH or NaOH, or acid hydrolysis, advantageously in the presence of an alcohol such as methanol or ethanol, or via enzymatic cleavage, and isolated via, for example, phase separation and subsequent acidification via, for example H_2SO_4. The fatty acids can also be liberated directly without the above-described processing step.

If further purification is necessary, standard methods can be employed. Such methods may include extraction, treatment with urea, fractional cyrstallization, HPLC, fractional distillation, silica gel chromatography, high-speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques (e.g. alkylation, iodination, use of butylated hydroxytoluene (BHT). Methods used include methylation of the fatty acids to produce methy esters. Similarly, protecting groups may be removed at any step. Desirably, purification of fractions containing, for example, ALA, STA, ETA,

EPA, DPA and DHA may be accomplished by treatment with urea and/or fractional distillation.

Large scale purification methods of fatty acids from algae are known in the art. For example, a microalgae strain is cultivated to increase cell density using photobioreactors, open ponds, race ways or hybrid systems. Algal cells are separated from culture media by filtration, flocculation or centrifugation, followed by drying to improve extraction. Lipid extraction is then commonly performed using a non-water miscible organic solvent. Larger scale extraction is typically carried out with hexane as a solvent. Subsequently, unsaturated fatty acids are separated from the total lipids by fractional (molecular) distillation or winterization, whereby oil temperature is reduced to precipitate the more saturated lipids. Further processing to improve the quality, shelf-life and quantity of PUFA oil can include filtration, bleaching, deodorization, polishing and antioxidant addition. These methods are all known to a person skilled in the art.

In another aspect, the invention also relates to the use of the transgenic organism, preferably microalgae, as described herein in the production of fatty acids, preferably a omega-3 fatty acids. The invention encompasses the use of a transgenic organism, preferably microalgae, as described herein or of the oil, lipid, the fatty acids obtained from a transgenic organism, preferably microalgae, as described herein in feedstuffs, foodstuffs, cosmetics, nutriceutical or pharmaceuticals. The invention encompasses the use of a transgenic organism, preferably microalgae as described herein, in producing feedstuffs, foodstuffs, cosmetics, nutriceutical or pharmaceuticals. In another aspect, the invention also relates to the use of the transgenic microalgae, as described herein as a feedstuff for animals, preferably fish.

In another aspect, the invention also relates to a composition comprising the transgenic microalgae as described herein or a fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said microalgae. In a preferred embodiment, the composition comprises the transgenic microalgae as described herein or a product obtained or obtainable therefrom., such as an oil. In one embodiment, the composition may be a pharmaceutical composition, a cosmetic, a foodstuff, including food supplements, or feedstuff for animals. In particular, the invention relates to a foodstuff comprising the transgenic microalgae as described herein or fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said algae. This can be in the form of a dietary

supplement, including fish oils. The invention also relates to an animal feed, especially for aquaculture, comprising the transgenic microalgae as described herein or fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said algae.

In another aspect, the invention relates to a composition comprising the transgenic microalgae as described herein, a fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said microalgae for use in medicine. In particular, the composition may be used to lower both blood pressure and heart rate in hypertensive individuals reducing the risk of sudden death, reduce inflammation, and to reduce the long-term risk of atherosclerosis and ischemic heart disease. The composition may also be used to treat eczema or metabolic syndrome. Also, a DHA rich diet is associated with increased cognitive abilities and depression and has a positive effect on arthritis and type II diabetes (Horrocks et al, 1999). Thus, the invention also relates to a composition comprising the transgenic microalgae as described herein or fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said microalgae for use in the treatment or prevention of cardiovascular conditions, including atherosclerosis, thrombosis, high blood pressure, myocardial infarction and atherosclerosis, inflammatory conditions, depression, cognitive decline, arthritis, and type II diabetes. Also encompassed in the scope of the invention are methods of treating or preventing cardiovascular and inflammatory conditions, depression, cognitive decline, arthritis and type II diabetes administering a composition comprising a therapeutic amount of the transgenic microalgae as described herein, a fatty acid, preferably a omega-3 fatty acid, oil, or lipid obtained from said microalgae to a patient in need thereof. The invention also relates to the use of a composition comprising the transgenic microalgae as described herein in the manufacture of a medicament for treating cardiovascular conditions, including atherosclerosis, thrombosis, high blood pressure, myocardial infarction and atherosclerosis, inflammatory conditions, depression, cognitive decline, arthritis, and type II diabetes.

In preferred embodiments, the composition may comprise or be obtained from a transgenic microalgae expressing a nucleic acid encoding a Δ6-desaturase and/or a transgenic microalgae expressing a nucleic acid encoding a Δ5-elongase as described herein.

The inventors have shown that microalgae can be manipulated using recombinant methods to produce an increased amount of LC-PUFAs, in particular EPA and DHA using heterologous gene expression. The inventors have surprisingly demonstrated that heterologous expression of Δ5-elongase from *Ostreococcus tauri* alone results in increased accumulation of DHA in *P. tricornutum* with DHA levels in transgenic strains reaching up to 13% of total fatty acids. A skilled person would understand that the invention is not restricted to algae and can indeed be applied to any organism that makes EPA/DHA. Thus, the invention also relates to a transgenic organism with increased DHA levels expressing a heterologous Δ5-elongase, preferably a Δ5-elongase from *Ostreococcus tauri.* In one embodiment, no other transgenes are expressed in the transgenic organism. In another embodiment, further transgenes may be expressed as described herein. Furthermore, the invention also relates to methods for increasing the production of DHA in a transgenic organism. This is achieved by expressing a heterologous Δ5-elongase, preferably a Δ5-elongase from *Ostreococcus tauris* in said organism. Details of said methods are described herein.

The organism may be an animal, for example a mammal. In one embodiment, humans are specifically excluded. In another embodiment, the organism is a plant, for example a monocot or dicot plant, for example crop plant. Crop plants include but are not limited to maize, rice, wheat, oilseed rape/canola, sorghum, soybean, sunflower, alfalfa, potato, tomato, tobacco, grape, barley, pea, bean, field bean, lettuce, cotton, sugar cane, sugar beet, broccoli or other vegetable brassicas or poplar.

In another aspect, the invention relates to isolated nucleic acids encoding for novel forms of the desaturases and elongases which may be useful in the heterologous reconstitution of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway in algae and higher plants. Specifically, the invention relates to isolated nucleic acids encoding Δ6-desaturase (Ost809Δ6), Δ4-desaturase (Ost809Δ4) and Δ6-elongase (FcELO6) and their corresponding polypeptides.

In one embodiment, the invention relates to an isolated nucleic acids comprising SEQ ID No. 7 or 9 encoding Δ6-desaturase (Ost809Δ6) comprising or consisting of SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%,

at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 10. The sequence may also be codon optimised for expression the target organism.

In one embodiment, the invention relates to an isolated nucleic acid comprising SEQ ID No. 15 or 17 encoding a Δ4-desaturase (Ost809Δ4) comprising or consisting of SEQ ID No.16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18. The sequence may also be codon optimised for expression the target organism.

In one embodiment, the invention relates to an isolated nucleic acid comprising SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20. The sequence may also be codon optimised for expression the target organism.

In one embodiment, the invention relates to an isolated nucleic acid comprising SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22. The sequence may also be codon optimised for expression the target organism.

The invention also relates to a vector comprising one or more of the isolated nucleic acids as specified above. The vector may further comprise a regulatory sequence.

The invention also relates to a transgenic microalgae with increased production of omega-3 LC-PUFAs wherein said microalgae expresses a nucleic acid comprises SEQ ID No. 7, 9, 15, 17, 19 or 21 or a sequence that encodes for a peptide that has at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%

homology to SEQ ID No. 8, 10, 16, 18, 20 or 22. Compositions comprising the transgenic microalgae, oil or lipids isolated therefrom and uses of as described herein in medicine or the formulation of a medicament, methods of treatment or feedstuff, foodstuff, pharmaceuticals or nutriceutical are also within the scope of the invention.

Without wishing to be bound by theory, the inventors believe that the activities of these nucleotides will prove useful in the heterologous reconstitution of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway in algae and plants. For example, the superior substrate-preference of the Ost809Δ6 enzyme distinguishes it from other *Ostreococcus* D6-desaturases, and can be used to maximise the flux of substrate through the n-3 pathway. Similarly, the Ost809Δ4 activity will prove useful in the specific conversion of DPA to DHA in transgenic photosynthetic organisms, whilst the FcELO6 activity provides a means by which GLA can be elongated to 20:3n-6.

In another embodiment, the invention therefore relates to the use of an isolated nucleic acid selected from a nucleic acid comprising or consisting of SEQ ID No. 7 or 9 encoding Δ6-desaturase (Ost809Δ6) comprising or consisting of SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 10, a nucleic acid comprising or consisting of SEQ ID No.16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18, a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least

96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22 in the production of a transgenic organism with increased omega-3 fatty acid content. In particular, the invention relates to the use of isolated nucleic acids encoding a Δ6-desaturase (Ost809Δ6) to maximise the flux of substrate through the n-3 pathway and produce enhanced levels of EPA and/or DHA. In another embodiment, the invention relates to the use of an isolated nucleic acid encoding a Δ4-desaturase (Ost809Δ4) to convert DPA to DHA. In another embodiment, the invention relates to the use of an isolated nucleic acid encoding a Δ6-elongase to elongate GLA to 20:3.

In another embodiment, the invention relates to the use of an isolated nucleic acid selected from a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22 in increasing DHA content. As shown in the examples and figure 13, DHA is increased by at least 10%, for example 14-17%.

In another embodiment, the invention relates to a method for producing a transgenic organism with increased of omega-3 LC-PUFAs production, in particular DHA and/or EPA, comprising transforming an organism with an isolated nucleic acid comprising or consisting of SEQ ID No. 7 or 9 encoding Δ6-desaturase (Ost809Δ6) comprising or consisting of SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 10, a nucleic acid comprising or consisting of SEQ ID No.16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least

85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18, a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22.

In one embodiment, the invention relates to a method for producing a transgenic organism with increased of DHA production, comprising transforming an organism with an isolated nucleic acid nucleic acid selected from a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22 in increasing DHA content. As shown in the examples and Figures 13, DHA is increased by at least 10%, for example 14-17%.

In another embodiment, the invention relates to a method for increasing the production of omega-3 fatty acid transforming an organism with an isolated nucleic acid comprising or consisting of SEQ ID No. 7 or 9 encoding Δ6-desaturase (Ost809Δ6) comprising or consisting of SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-

desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 10, a nucleic acid comprising or consisting of SEQ ID No.16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18, a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22.

In one embodiment, the invention relates to a method for increasing the production of omega-3 fatty acid transforming an organism with an isolated nucleic acid nucleic acid selected from a nucleic acid comprising or consisting of SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising or consisting of SEQ ID No. 20, a functional variant thereof or a Δ6-elongase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20 or a nucleic acid comprising or consisting of SEQ ID No. 21 encoding a Δ5-desaturase comprising or consisting of SEQ ID No. 22, a functional variant thereof or a Δ5-desaturase that has at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%,at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22 in increasing DHA content. As shown in the examples and Figures 13, DHA is increased by at least 10%, for example 14-17%.

In one embodiment of the methods, method may further comprise transforming said microalgae with one or more additional nucleic acid that regulates the production of omega-3 fatty acids. In another embodiment, no additional nucleic acid that regulates the production of omega-3 fatty acids are introduced into said microalgae. Other heterologous nucleic acids, for example encoding a glucose transporter may be included.

In another aspect, invention relates to a host cell transformed with a vector comprising one or more of the isolated nucleic acids defined herein, specifically an isolated nucleic acid comprising SEQ ID No. 1, 3, 5, 7, 9, 15, 17, 19 or 21. In one embodiment, the host cell is transformed with a vector comprising one of the isolated nucleic acids defined herein and no other heterologous transgenes involved in the regulation of the LC-PUFAs biosynthetic pathway are expressed in said organism.

The host cell may be an algae or a higher plant cell. For example, the host cell is a microalgae. In one embodiment, the host cell is a diatom. The host cell may also comprise one or more additional transgene. For example, the host cell may be a transgenic microalgae described herein expressing a nucleic acid encoding for a Δ5-elongase.

The transgenic organism according to the methods described above may a microalgae or a higher plant. Preferably, the transgenic organism according to the methods described is a microalgae. The term microalgae is defined elsewhere herein and includes a diatom. In one embodiment, the microalgae is *P. tricornutum*. The term higher plant includes monocot and dicot plants. In one embodiment, the plant is a crop plant as described herein.

All references cited in this disclosure are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this application.

"and/or" where used herein is to be taken as specific disclosure of each of the multiple specified features or components with or without the other at each combination unless otherwise dictated. For example "A, B and/or C" is to be taken as specific disclosure of

each of (i) A, (ii) B, (iii) C, (iv) A and B, (v) B and C or (vi) A and B and C, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

The invention is further described in the following non-limiting examples.

Examples

Example 1 Generation of transgenic algae over-expressing Δ6-desaturases and Generation of transgenic algae over-expressing Δ5-elongase

Materials and Methods

Strains and growth conditions

P. tricornutum UTEX 646 was grown in ESAW medium (Harrison et al., 1980) at 18°C and 20°C with moderate shaking under white fluorescent lights in constant illumination (30 µmol and 60 µmol photons m- $^{2}s^{-1}$). Analysis of the wild-type and transgenic algae have been performed during exponential and stationary growth phases.

Plasmid design and cloning

The coding sequences for Δ6 – desaturase from *Ostreococcus tauri* , OtD6 (Domergue et al., 2005) and *O.tauri* Δ5- elongase OtElo5 (Meyer et al., 2004) were inserted as *Kpn-Xba* and *EcoRV-SacI* fragments, respectively, into pPha-T1 vector (Zaslavskaia et al., 2000), kindly provided by Dr. P.G.Kroth, (Universitat Konstanz, Germany). The coding region of OtD6 was used as a template to chemically synthesize (Genscript Corporation, NJ) codon-optimized nucleotide sequence OtD6PT for expression in *P.tricornutum*. This codon-optimized Δ6 – desaturase sequence was cloned into pPha-T1 vector, using *EcoRV-SacI* sites. The coding sequences for Δ6 – desaturase from *P. tricornutum*, PtD6 (Domergue et al., 2002) was inserted as *BamHI –XbaI* fragment into pPha-T1 vector (Zaslavskaia et al., 2000).

Biolistic transformation

Biolistic transformation of *P. tricornutum* was performed according to previously described (Zaslavskaia et al., 2000; Kroth 2007). Bombarded cells were transferred onto ESAW agar plates containing 75 µg/ml zeocin. The zeocin plates were placed in 24 h light under fluorescent lights (50 µmol m^{-2} s^{-1}) and incubated at 20°C for 3 weeks. Selected zeocin-resistant colonies were transferred to fresh zeocin plates and 2 ml ESAW+ zeocin cultures before being transferred to liquid medium minus antibiotic for lipid analysis.

Fatty acid analysis

Algae or yeast cells were harvested by centrifugation. Fatty acids were extracted and methylated as described (Garces and Mancha, 1993) with minor modifications. A 15ml aliquot of algal culture was harvested; following methylation the heptane fraction was concentrated and re-suspended in 40 µl solvent prior to injection of 1 µl on to the GC column. Methyl ester derivatives of total fatty acids extracted were analysed by GC using an Agilent DB-225 column and identified using known standards.

Acyl-CoA profiling

Algal cells were harvested by centrifugation, frozen in liquid nitrogen and extracted after Larson and Graham (2001), for reverse-phase LC with either quantitative analysis of fluorescent acyl-etheno-CoA derivatives or with electrospray ionization tandem mass spectrometry (multi reaction monitoring) in positive ion mode For the analysis of etheno-CoA derivatives HPLC (Agilent 1200 LC system; Phenomenex LUNA 150 · 2 mm C18(2) column) was performed using the methodology and gradient conditions described previously (Larson and Graham 2001); whilst LC-MS/MS +MRM analysis followed the methods described by Haynes et al. 2008 (Agilent 1200 LC system; Gemini C18 column, 2 mm inner diameter,150 mm with 5 mm particles). For the purpose of identification and calibration, standard acyl-CoA esters with acyl chain lengths from C14 to C20 were purchased from Sigma as free acids or lithium salts.

Lipid Profiling

The molecular species of TAGs and PLs were analysed by electrospray ionisation triple quadrupole mass spectrometry (API 4000 QTRAP; Applied Biosystems). The molecular species of polar lipid were defined by the presence of a head-group fragment and the mass/charge of the intact lipid ion formed by ESI (Welti et al., 2002; Devaiah et al., 2006 with modifications described by Xiao et al. 2010). Such tandem ESI-MS/MS

precursor and product ion scanning, based on head group fragment, do not determine the individual fatty acyl species. Instead, polar lipids are identified at the level of class, total acyl carbons, and total number of acyl carbon–carbon double bonds. Polar lipids were quantified in comparison with a series of polar lipid internal standards. Triacylglycerols (TAGs) measured after Krank et al. (2007) were defined by the presence of one acyl fragment and the mass/charge of the ion formed from the intact lipid (neutral loss profiling). This allows identification of one TAG acyl species and the total acyl carbons and total number of acyl double bonds in the other two chains. The procedure does not allow identification of the other two fatty acids individually nor the positions (sn-1, sn-2, or sn-3) that individual acyl chains occupy on the glycerol. TAGs were quantified in a manner similar to the polar lipids, including background subtraction, smoothing, integration, isotope deconvolution and comparison of sample peaks with those of the internal standard (using LipidView, Applied Biosystems). However, whereas polar lipids within a class exhibit similar mass spectral response factors, the mass spectral responses of various TAG species are variable, owing to differential ionization of individual molecular TAG species. In the data shown herein, no response corrections were applied to the data. The data were normalized to the internal standards tri15:0 and tri19:0

Results

Generation of transgenic algae over-expressing Δ6-desaturases.

The native coding OtD6 and codon-optimized for expression in *P. tricornutum* nucleotide sequences for *O.tauri* Δ6 – desaturase were cloned into pPha- T1 vector, generating expression cassettes OtD6N and OtD6Pt respectively, and the resulted constructs were used to transform *P. tricornutum.*

Expression of OtD6N construct

13 zeocin resistant colonies were obtained by transformation with OtD6N and selected for further screening. Selected colonies were transferred into liquid medium and several positive transformants containing OtD6N were identified. We have studied the effects of temperature and light on the production of EPA and total fatty acids in Wt and transgenic *P.tricornutum.* Cultures were grown at different temperatures (18^{o}C and 20^{o}C) under constant illumination in different light intensity (25 µmol and 60 µmol photons m- $^{2}s^{-1}$). GC-MS analyses have been performed during the exponential (E) and stationary (S) phases of cell growth. Fatty acid profiling of WT and mutants showed

that palmitoleic acid (16:1Δ^9), EPA (20:5 n-3), palmitic acid (16:0) and myristic acid (14:0) were the major FAs detected in algal cells grown in both stages. Similarly to the results obtained by Tonon et al. (Tonon 2002) from the studies of *P. tricornutum* (CCAP 1052/1A) cell cultures grown at 18°C with 240 µE m- ^{2}s^{-1} , there was decrease in the amount of EPA and DHA as the cells of *P.tricornutum* UTEXS 646 used in our study shifted from exponential to stationary phase . Fatty acid analysis revealed that in cells transformed with Otd6N and grown at 20°C in light intensity 25 µmol and 60 µmol photons m- ^{2}s^{-1} EPA and DHA decreased upon transition to stationary phase. However, the levels of EPA and DHA in Otd6N cells grown at 20°C, 60 µE m- ^{2}s^{-1} in stationary phase were higher than those of WT *P. tricornutum* (21.2% of EPA and 1.8% of DHA in Otd6N compared to 18.5% of EPA and 1.3% of DHA in WT (Table III, Fig. 1). In contrast, we found that in transgenic Otd6N cells grown at 18°C, 25 µE m- ^{2}s^{-1} levels of EPA and DHA increased in stationary phase compared to exponential phase and are significantly higher than in WT samples (30.2% of EPA and 1.8% of DHA in Otd6N compared to 16.5% of EPA and 0.9% of DHA in WT). Fatty acids profiles from Wt and Otd6N transgenic P. *tricornutum* showed no differences in Δ6 – unsaturated fatty acids (GLA and SDA) composition, which were barely present.

Expression of OtD6PT construct

4 zeocin resistant colonies obtained by transformation with OtD6PT were selected to inoculate cultures for further screening and GC-MS analysis. The same trend towards decreasing levels of EPA and DHA in the stationary phase was observed for transgenic Otd6Pt cells grown at different light intensity and temperatures (Table III, Fig.1). Recombinant cells expressed higher levels of EPA (20.8% in the stationary phase at 20°C, 60 µE m- ^{2}s^{-1} and 22.2% at 18°C, 25 µE m- ^{2}s^{-1} compared to 18.5% and 16.8% in WT respectively). In addition to detection of higher levels of EPA we also observed an increase in DHA levels with minor variation between the two phases of growth (Table III, Fig.1).

Generation of transgenic algae over-expressing OtElo5

3 zeocin resistant clones obtained by transformation with OtElo5 were identified in an initial screen and used to inoculate cultures for further screening and GC-MS analysis. Cultures were grown at 20°C under constant illumination in 60 µmol photons m- ^{2}s^{-1}. FAMEs analysis of *P. tricornutum* transformed with OtElo5 have been performed during the exponential (E) and stationary (S) phases of cell growth and revealed the presence

of DPA in the range of 2.8-4.7% in transgenic clones which was not detected in WT cells (Table IV, Fig 2a). Levels of EPA in transformed clones were decreased to an average of 17.7% compared to 35.9% in WT in the exponential phase of growth and to 8.2% in clones over-expressing the Elo5 gene compared to 18.5% in WT during the stationary phase of growth. A substantial increase in DHA was observed in all 3 transgenic clones averaging 7.4% in exponential phase and 10.4% in stationary phase compared to 2.0% and 1.3% respectively in WT. DHA accumulation has been increased upon transition to stationary phase.

Determination of acyl-CoA pool composition

To better understand the processes of acyl desaturation in diatoms the composition of the acyl-CoA pool was determined for the wild–type (WT) and transgenic *P. tricornutum*, expressing OtElo5-elongase (Fig.3). The study of acyl-CoA profile of WT *P. tricornutum* in the stationary phase of growth revealed that palmitic, palmitoleic, stearic, oleic and EPA-CoA were the most abundant, thus demonstrating the direct relationship between the levels of native fatty acids in the acyl-CoA pool vs the total fatty acids. EPA-CoA represented 5.7% of the acyl-CoA pool, indicating that this level of EPA-CoA could potentially act as an intermediate in the synthesis of DHA through elongation to 22:5n-3 and desaturation to 22:6n-3. Only traces (<1.0) of 22:4 n-6, 22:5 n-3 (DPA) and DHA were detected in the CoA pool of WT *P. tricornutum*. As can be seen in Figure 3, similar analysis of transgenic *P. tricornutum* demonstrated a significant increase in the levels of 22:4 n-6, 22:5 n-3 (EPA) and DHA accompanying by the decrease in EPA levels. As shown in Figure 4, detailed analysis of the composition of the acyl-CoA pool through different stages of cell growth revealed that EPA and DHA were accumulating progressively from exponential to stationary phase displaying maximum levels of 5.2% and 6.3% in stationary phase.

Profiling of TAG molecular species

In this study we identified and compared the molecular species of TAGs formed by WT and OtElo5 transgenic *P. tricornutum* and investigated changes in TAG synthesis in response to transition from exponential to stationary phase. Cultures were grown at 20^{o}C under constant illumination in 60 µmol photons m- $^{2}s^{-1}$ and analysed using ESI-MS. The mass spectrum obtained from direct infusion ESI-MS of algal lipid extracts shows that a majority of the molecular ions are observed between 750 and 950 mass/charge (m/z). We detected 26 individual TAG species in WT *P. tricornutum.* The

oil extracts of WT were predominantly composed of TAGs 46:1, 46:2 48:1, 48:2, and 48:3 and 50:3, having palmitic (16:0), palmitoleic (16:1), and myristic (14:0) acid substituents. TAG 48:1 (16:0/16:0/16:1) and 48:2 (16:0/16:1/16:1) constitute the main TAG molecular species that is expressed throughout the time course analysis of *P. tricornutum* cells (Figs 5a and 5B). An increase in the diversity of TAG molecular species (with as much as 29 individual TAGs) was detected from cells expressing OtElo5 –elongase. Specifically, new TAG species, 54:8, 54:9 and 56:8 were observed and transgenic cells show significantly higher levels of 54:7. DHA was incorporated in TAGs 52:7, 54:7, 54:8, 54:9 and 56:8. The time course (Fig. 6) also revealed that TAGs 54:7 and 56:8 appear to have more DHA incorporated into TAGs as the cells shift from the exponential growth phase to the stationary phase. TAGs molecular species 52:7, 54:8 and 54:9 demonstrated more or less constant DHA proportions when cultures were shifted from exponential to stationary phase. Levels of TAGs containing DHA averaged 12.5% in exponential stage and 10.5% in the stationary phase.

Table III. Fatty acid composition (molar %) of WT and transgenic *P. tricornutum* expressing *O. tauri* Δ6 desaturase under different growth conditions at two growth stage, where E is the exponential and S is the stationary growth phases. Each measurement is the average of three biological replicates.

Cell strain	20°C 60µmol photons		20°C 25µmol photons		18°C 25µmol photons	
	E	S	E	S	E	S
Otd6N 14:0	6.3±1.1	5.6±1.6	11.5±0.7	7.6±1.5	13.0±1.1	10.9±1.0
16:0	16.0±0.5	21.0±1.3	12.8±0.9	16.8±1.6	15.3±0.8	16.6±1.1
16:1	28.3±1.7	36.5±1.6	32.8±0.2	30.3±1.9	35.1±2.1	34.4±2.5
16:3	2.5±0.2	0.9±0.2	4.0±0.6	0.9±0.1	3.6±0.0	2.7±0.2
18:0	0.5±0.0	0.7±0.0	0.3±0.0	0.4±0.0	ND	ND
18:1	6.2±1.4	8.6±1.5	18.1±0.0	24.9±0.3	2.1±0.2	2.5±0.2
18:2n-6	1.5±0.1	0.6±0.0	ND	ND	1.4±0.2	1.4±0.2
18:3 n-6	0.7±0.3	1.3±0.3	ND	ND	ND	ND
18:4 n-3	0.8±0.1	0.8±0.1	ND	0.4±0.0	1.0±0.4	1.0±0.4
20:5 n-3	32.2±3.6	21.2±1.9	20.6±1.1	17.8±2.6	27.1±2.7	30.2±3.2
22:6 n-3	2.3±0.2	1.8±0.3	1.4±0.1	1.0±0.1	1.4±0.4	1.8±0.3
Others	6.89±0.6	4.3±0.6	12.2±1.8	6.0±0.2	5.7±0.4	6.2±0.6

Otd6Pt	14:0	7.0±1.4	4.9±1.0	5.6±0.2	4.9±0.2	12.8±0.1	7.4±0.4
	16:0	16.3±1.3	20.2±1.5	9.5±0.3	16.8±0.7	17.0±0.9	20.4±0.2
	16:1	27.1±4.0	38.6±3.6	24.5±0.2	33.4±7.9	28.3±1.2	35.8±2.6
	16:3	2.5±0.2	1.1±0.3	4.0±0.6	1.4±0.1	2.9±0.0	5.2±1.1
	18:0	0.5±0.1	0.6±0.1	0.3±0.0	0.4±0.0	ND	ND
	18:1	7.8±0.2	8.7±0.4	26.9±5.4	24.9±0.3	6.0±0.9	8.5±0.9
	18:2 n-6	1.1±0.2	1.1±0.1	ND	ND	1.2±0.0	1.2±0.0
	18:3 n-6	1.2±0.2	0.8±0.0	0.2±0.0	0.2±0.0	ND	ND
	18:4 n-3	1.1±0.1	1.2±0.1	0.6±0.1	0.6±0.0	1.5±0.0	1.5±0.0
	20:5 n-3	33.2±1.4	20.8±3.5	27.0±4.0	16.6±2.0	25.8±0.1	22.2±1.3
	22:6 n-3	1.7±0.3	1.5±0.4	1.3±0.1	1.2±0.6	1.1±0.0	1.3±0.2
	Others	9.2±0.6	4.3±0.9	12.3±1.8	5.5±3.6	7.3±0.3	3.1±0.3
WT	14:0	7.7±0.5	4.8±0.1	5.1±0.2	4.8±0.5	10.9±0.5	7.9±0.1
	16:0	16.5±0.4	22.2±0.6	11.0±2.0	16.6±3.2	19.7±0.4	21.1±1.3
	16:1	28.4±0.6	41.8±0.5	22.3±1.1	32.2±4.1	35.8±0.6	42.1±2.5
	16:3	2.4±0.3	1.0±0.1	2.6±0.6	0.6±0.1	2.4±0.3	1.4±0.0
	18:0	0.4±0.0	0.5±0.0	0.3±0.1	0.3±0.1	ND	ND
	18:1	3.8±0.8	7.3±0.2	28.9±1.4	25.7±4.9	6.1±0.3	8.2±0.1
	18:2n-6	1.4±0.1	0.6±0.0	ND	ND	1.1±0.1	0.8±0.1
	18:3n-6	0.7±0.0	0.6±0.0	ND	ND	ND	ND
	18:4 n-3	0.8±0.0	1.0±0.0	0.6±0.0	0.4±0.1	1.0±0.7	0.6±0.8
	20:5n-3	35.9±1.6	18.5±0.4	27.6±2.3	17.1±2.5	22.2±0.7	16.8±2.8
	22:6n-3	2.0±0.3	1.3±0.0	1.8±0.1	1.3±0.3	0.8±0.1	0.9±0.2
	Others	6.8±0.3	2.4±0.3	10.0±0.9	5.1±0.8	4.9±0.5	2.9±0.3

Table IV. Fatty acid composition (molar %) of WT and transgenic *P. tricornutum* expressing Ot Elo5 during exponential (E) and stationary (S) phases. Cultures were grown at 20°C 60 µmol $m^{-2}s^{-1}$ under constant agitation at 70 rpm. Each measurement is the average of 3 biological replicates.

Fatty acids	WT		OtElo5	
	E	S	E	S
14:0	7.7±0.5	4.8±0.5	8.4±1.2	5.3±1.6
16:0	16.5±0.5	22.1±0.6	16.8±0.6	17.4±1.3
16:1	28.4±0.6	41.8±0.5	32.9±0.4	42.5±1.6
16:3	2.4±0.3	1.0±0.0	3.6±0.6	1.7±0.6
18:0	0.4±0.0	0.5±0.0	0.6±0.0	0.5±0.0
18:1	3.8±0.8	7.3±0.2	6.8±1.1	6.8±1.5
18:2 n-6	1.4±0.1	0.6±0.0	0.6±0.0	0.3±0.0
18:3n-6	0.7±0.0	0.6±0.0	0.2±0.0	0.2±0.2
18:4 n-3	0.8±0.0	1.0±0.0	1.6±0.0	2.0±0.1
20:5 n-3	35.9±1.6	18.5±0.4	17.7±2.4	8.2±2.0
22:5 n-3	ND	ND	3.3±0.5	3.4±1.2
22:6 n-3	2.0±0.3	1.3±0.1	7.4±1.2	10.4±0.3
24:0	5.2±0.2	2.1±0.0	5.2±0.4	3.1±0.4
Others	1.8±0.3	0.3±0.3	4.1±0.4	2.4±0.6

Discussion

Many marine microbes produce high levels of EPA and DHA but only few species have the ability to partition these fatty acids into storage lipids in the form of triacylglycerols (TAGs). The majority of algal species accumulate saturated and mono-unsaturated fatty acids in TAGs (Harwood, 1998; Roessler, 1990b). Partitioning of LC-PUFAs into TAGs have been observed in *Parietochloris incise* (Bigogno et al., 2002), the freshwater red microalga *Porphyridium cruentum* (Cohen et al., 2000), and marine microalgae *Nannochloropsis oculata*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana and Pavlova lutheri*, (Tonon et al., 2002). Thus these species are good candidates for further studies, in order to understand the processes responsible for the incorporation of LC-PUFAs into storage oils in microalgae.

At present it is generally accepted that oleaginous algae produce small quantities of TAG under optimal growth conditions (Hu et al. 2008). Among major factors affecting triacylglycerol accumulation and fatty acid composition in microalgae are temperature and light intensity. Generally, it is considered that fatty acid unsaturation increases with temperature decrease and low light favours the formation of PUFAs. For example, in *P.tricornutum* UTEXS 640 optimal culture temperature for EPA production was 21.5 to 23°C (Yongmanitchai W. and Ward O., 1991). A temperature shift strategy has been

employed to enhance the overall n-3 PUFAs (including EPA) production because the optimal temperature for microalgal growth is often higher than that for n-3 PUFAs formation (Jiang and Chen, 2000). Such a phenomenon has been observed in many different algal species including *P. cruentum* (Springer et al., 1994), *Nannochloropsis* sp. (Sukenik, 1991) and *P. irregular* (Stinson et al., 1991). However, Ohta et al. (1993) observed that the optimal temperature for growth of *P. purpureum* also yields a biomass with the highest EPA content. These results suggest that the effect of temperature on cell growth and n-3 PUFA production should be carefully studied for individual microalgal species.

Profiling of TAG species in *P. tricornutum* has been previously reported (Yongmanitchai and Ward 1993; Yu et al., 2009). We observed the same predominant fatty acids (i.e., 14:0, 16:0, 16:1, 16:3, and 20:5) incorporated in TAGs as described in these earlier studies. Yongmanitchai and Ward 1993 identified only 18 TAG molecular species via reverse-phase HPLC analysis. Due to the high resolution and sensitivity of ESI-MS, Yu et al., 2009 were able to detect twofold more species in algal oil extracts (14 of the 18 species they detected by HPLC, at comparable percentage composition. However, TAGs 48:7, 48:9, 48:12, and 54:10 were not detected which could be explained by the difference in the *P. tricornutum* strains and culture conditions.

Example 2

Identification and characterization of new activities for PUFAs biosynthesis in algae and plants

2.1 Identification of a Δ6 – desaturase from the microalga *Ostreococcus* RCC809

Genome of green alga *Ostreococcus* RCC809 was analysed with BLAST using already known N-terminal cytochrome b5-fusion desaturases as query. This analysis revealed the presence of several genes coding for putative PUFA desaturases. The deduced open reading frames were used as templates to chemically synthesise (Genscript Corporation, NJ) codon-optimised nucleotide sequences for expression in diatoms.

Functional characterization of putative Ostreococcus RCC809 Δ6 –desaturase in yeast.

The codon-optimised open reading frame of the putative Δ6 – desaturase (SEQ ID No.s 7 to 10, hereafter designated Ost809Δ6) was inserted as *KpnI-SacI* fragment behind the galactose –inducible GAL1 promoter of the yeast expression vector pYES2 (Invitrogen, NJ). Ost809Δ6

The *S. cerevisiae* strain W303-1A was transformed with plasmid DNA using a lithium acetate method. Cultures were grown at 22°C in the presence of 2% (v/v) raffinose for 48 h, and expression of the transgene was induced by addition of galactose to 2% in the presence of 0.5 mM of linoleic acid (LA, 18:2n-6) and 1% (w/v) tergitol NP-40 (Sigma) as described (Sayanova et al., 2001).

The predicted function of the candidate desaturase Ost809Δ6 (predicted to encode a C18 Δ6-desaturase of 461 amino acids) was investigated by expression studies in *S. cerevisiae* in the presence of a range of potential fatty acid substrates. Total fatty acid methyl esters from yeast cells were then analysed by GC–FID and the identity of novel peaks confirmed by GC–MS and co-migration with authentic standards. As shown in Fig. 8, expression of a synthetic ORF encoding Ost809Δ6, confirmed the enzymatic capability to convert exogenously supplied substrate (α-Linolenic acid, ALA; C18:Δ9,12,15) to the Δ6-desaturated product SDA (18:4, n-3). In the absence of galactose, the exogenous substrate ALA is not converted to SDA. Thus, on the basis of these results, Ost809Δ6 was confirmed as a D6-desaturase. The substrate selectivity of Ost809Δ6 was determined by exogenously supplying equal quantities of LA and ALA in the growth media. As it is shown in Figure 9, Ost809Δ6 only recognised the n-3 fatty acid ALA as a substrate, whereas the n-6 substrate was not desaturated. This is distinct from a Δ6-desaturase identified from *Ostreococcus tauri* (Domergue et al, 2005), which showed activity towards both LA and ALA as substrates. Thus Ost809Δ6 is superior and distinct for the exclusive production of Δ6-desaturated n-3 fatty acids.

Yeast cultures were supplemented with different potential FA substrates (listed in Table V) but desaturation activity of O809d6 was detected only in the presence of ALA.

2.2 Identification of putative Δ4 – desaturase from O809

The genome sequence of *Ostreococcus* RCC809 http://genome.jgi-psf.org/OstRCC809_2/OstRCC809_2.home.html was searched with previously functionally characterised sequences of Δ4–desaturases and the presence of an apparent candidate (JGI protein ID # 40461) for a Δ4–desaturase was detected. The deduced open reading frame was used as a template to chemically synthesise (Genscript Corporation, NJ) codon-optimised nucleotide sequences for expression in diatom *P. tricornutum* (SEQ ID No.s 15 to 18).

Functional characterization of putative Δ4 – desaturase from O809 in yeast.

The codon-optimised for expression in *P. tricornutum* open reading frame of the putative Δ4 – desaturase was inserted as *KpnI-SacI* fragment behind the galactose – inducible GAL1 promoter of the yeast expression vector pYES2 (Invitrogen, NJ).

As can be seen in Fig 10, galactose-dependent expression of the Ost809 protein 40461 resulted in the Δ 4-desaturation of DPA to DHA, confirming the function of this ORF as a C22 Δ 4-desaturase and on this basis we designated this gene as Ost809Δ4. Note that in the absence of the inducer (galactose), no DHA is detected, nor in the absence of the Ost809Δ4 ORF.

2.3 Identification of a Δ6-elongase from *Fragilariopsis cylindrus*

The publically available genome sequence of the marine diatom *Fragilariopsis cylindrus* (http://genome.jgi-psf.org/Fracy1/Fracy1.home.html) was analysed with BLAST using already known Δ6-elongase sequences (such as the Δ6-elongase from C.elegans – Beaudoin et al, 2000) as query and a candidate open reading frame (designated Frag #177742) was used as a template to chemically synthesise (Genscript Corporation, NJ) codon-optimised nucleotide sequence for expression in *T.pseudonana*.

Functional characterization of Fc Δ6-elongase in transgenic yeast

Heterologous expression of Frag #177742 in *S. cerevisiae* was carried out exactly as described above, with the codon-optimised ORF cloned into the yeast expression vector pYES2. Galactose-mediated induction of this construct was used to confirm that this ORF functioned as a Δ 6-elongase, specifically elongating C18 Δ 6-unsaturated substrates such as GLA to a C20 form. As can be seen in figure 11, elongation of GLA to 20:3 only occurs in the presence of galactose and the ORF Frag #177742. On the basis of these results, this was redesignated FcELO6.

Table V. List of Substrates Tested:

Ost809 D6

18:2, ALA, GLA, 18:2 &18:3, 20:4n-6 (ARA), 20:2, ERA, ETA, 22:5n-6 (DPA)

FcElo6

18:2, GLA, GLA & SDA

Ost809Δ4

DPA

(Substrates underlined are those which worked)

Table VI. Fatty acid composition of yeast cells expressing Ost809Δ6, FcElo6 or Ost809Δ4 and substrate specificities of each of these

Fatty Acid Composition (molar %)										
	Construct									
FA	O809 Δ6 Gal -	O809 Δ6 Gal +	O809 Δ6 Gal -	O809 Δ6 Gal +	Fc Elo6 Gal -	Fc Elo6 Gal +	O809 d4 Gal -	O809 d4 Gal +	pYes2 BPX72	pYes2 HP1
16:0	26.2	26.0	24.8	22.4	25.2	23.2	22.8	20.4	26.1	22.2
16:1	25.6	28.8	26.3	27.9	23.7	26.3	49.2	51.0	29.2	51.5
18:0	ND	ND	ND	ND	ND	ND	4.2	4.4	ND	3.9
18:1	15.2	16.3	13.6	15.4	ND	ND	20.2	21.6	17.5	19.7
18:2	5.8	6.8	ND	ND	ND	ND	ND	ND	ND	ND
GLA	ND	ND	ND	ND	38.7	22.8	ND	ND	ND	ND
ALA	25.6	11.9	32.9	15.7	ND	ND	ND	ND	27.2	ND
SDA	1.6	10.3	2.3	18.5	ND	ND	ND	ND	ND	ND
DHGLA	ND	ND	ND	ND	ND	14.1	ND	ND	ND	ND
DPA	ND	ND	ND	ND	ND	ND	2.9	2.3	ND	2.7
DHA	ND	ND	ND	ND	ND	ND	ND	0.4	ND	ND

Table VII. Substrate specificity

Substrate Specificity		
Construct	**Substrate**	**%**
Ost809Δ6	18:2	0.0
Ost809Δ6	18:3 ALA	54.1
FcElo6	18:3 GLA	38.1
Ost809Δ4	22:5 DPA	13.5

On the basis of the identification of novel forms of the Δ 6-desaturase (Ost809Δ6), Δ 4-desaturase (Ost809Δ4) and the Δ 6-elongase (FcELO6), it is very likely that these activities will prove useful in the heterologous reconstitution of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway in algae and plants. For example, the superior substrate-preference of the Ost809Δ6 enzyme distinguishes it from other

Ostreococcus Δ 6-desaturases, and can be used to maximise the flux of substrate through the n-3 pathway. Similarly, the Ost809Δ4 activity will prove useful in the specific conversion of DPA to DHA in transgenic photosynthetic organisms, whilst the FcELO6 activity provides a means by which GLA can be elongated to 20:3n-3.

Example 3

Expression of single omega-3 LC-PUFA biosynthetic genes in Pheaodactylum tricornutum can increase the endogenous accumulation of DHA

Materials and methods

Strains and growth conditions

P. tricornutum UTEX 646 was grown in ESAW medium (Harrison et al., 1980) at 20°C with moderate shaking under white fluorescent lights in constant illumination (100 µmol photons m- $^{2}s^{-1}$). Analysis of the wild-type and transgenic algae have been performed during stationary growth phase.

Plasmid design and cloning

The coding sequence for Δ6-elongase FcElo6 (protein ID 177742) was used as a template to chemically synthesize (Genscript Corporation, NJ) a codon-optimized nucleotide sequencea for expression in *T. pseudonana*. The codon-optimized sequence was inserted as *EcoRV-SacI* fragments, respectively, into pPha-T1 vector (Kroth, 2007; Zaslavskaia et al., 2000).

Results

Expression of FcElo6 resulted in increase of DHA levels up to 14-17% (Figure 13).

Example 4

Co-expression of two genes

Material and methods

Design of double-gene vector pPhOS2 and transformation cassettes

The EcoRI –HindIII fragment of of pPha –T1 vector containing MCS was replaced by the synthetic sequence comprising of *fcpA* terminator and *fcpA* promoter flanked by 3 multiple cloning sites (MCSs) with unique restriction sites (Figure 14). The coding sequences for *O. tauri* Δ5-elongase OtElo5 was inserted as *KpnI-SacI* fragment into

position 1 of pPhOS vector generating pPhOS2.1.1 construct. The codon optimized for expression in *P.tricornutum* coding sequences for *O. tauri* Δ6–desaturase OtD6Pt was inserted as *BamHI- XbaI* fragment into position 2 of pPhOS2.1.1 generating pPhOS2.2.1 construct.

Results and Discussion

Multigene expression in transgenic P. tricornutum

To facilitate the expression of multiple heterologous genes in *P. tricornutum*, a new vector (designated pPhOS2- Figure 14) was constructed. This vector is based on previously described pPha-T1 vector (Zaslavskaia et al., 2000) and contains two multiple cloning sites (MCS) with unique restriction sites for inserting genes of interest. Each of these MCS is flanked by the promoter and terminator regions of the *FcpA* gene (Zaslavskaia et al., 2000) to promote the co-expression of two inserted genes. The coding sequence for *O. tauri* Δ5-elongase OtElo5 was inserted into position 1 of pPhOS2 vector and the resulting construct pPhOS2.1.1 was used to transform *P. tricornutum*. Cultures were grown at 20°C and 16°C under constant illumination (60 µmol photons $m^{-2}s^{-1}$). Multiple (5) independent zeocin-resistant colonies were obtained and used to inoculate cultures for further GC-MS analysis. The mean levels of DHA in analysed pPhOS2.1.1 strains was 9.0% (Table VIII; Figure 1), similar to levels previously observed with OtElo5 expression in pPHa-T1, confirming the functionality of this modified vector. The codon-optimized coding sequences for *O. tauri* Δ6–desaturase OtD6Pt was subsequently inserted into position 2 of construct pPhOS2.1.1, generating the two-gene (plus the selectable marker gene *ble*) pPhOS2.2.1 vector. This expression plasmid was introduced into *P. tricornutum* via biolistics and multiple independent zeocin-resistant colonies were obtained and used to inoculate cultures for further screening. Cultures were grown at 16 and 20°C under constant illumination (60 µmol photons m- $^{2}s^{-1}$). FAMEs analysis of transgenic strains expressing either single or double gene constructs revealed a further increase in DHA levels in transgenic strains co-expressing both OtElo5 and OtD6Pt, indicating the here-demonstrated potential for iterative metabolic engineering in *P. tricornutum* for high value lipid traits (Figure 15, Table VIII).

Table VIII. Fatty acid composition (Mol %) of wild-type (Pt_WT) and transgenic *P. tricornutum* expressing pPhOS2.1 and pPhOS2.2 at 16°C and 20°C. Each measurement is the average of 3 biological replicates (± Standard Error).

Fatty Acids	Pt_WT		pPhOS2.1		pPhOS2.2	
	16°C	20°C	16°C	20°C	16°C	20°C
	5.3±0.2	4.8±0.1	5.1±0.2	5.3±0.3	6.7±0.2	6.3±0.1
	22.3±1.0	22.1±0.4	19.2±0.4	18.9±1.4	17.7±0.5	18.4±0.3
14:0	39.2±1.6	41.8±0.3	39.0±0.6	40.1±1.7	43.6±1.0	40.6±0.5
16:0	0.8±0.4	1.0±0.1	1.2±0.1	1.8±0.4	nd	2.0±0.1
16:1	0.5±0.0	0.5±0.1	0.6±0.1	0.3±0.1	0.5±0.0	0.3±0.1
16:3	6.8±0.0	4.3±0.1	2.6±0.1	2.2±0.4	1.2±0.6	0.6±0.4
18:0	2.2±0.1	2.8±0.1	2.1±0.2	4.2±0.3	2.7±0.1	3.7±1.0
18:1 n-9	1.0±0.1	1.0±0.1	1.7±0.1	1.1±0.1	1.6±0.0	1.1±0.1
18:1 n-11	20.3±1.9	18.5±0.1	10.4±0.3	9.8±1.0	10.0±0.4	8.2±0.1
18:4 n-7	*nd*	*nd*	3.4±0.4	1.9±0.3	5.5±0.1	2.2±0.3
20:5 n-3	1.5±0.2	1.3±0.1	9.0±0.3	9.4±1.0	10.3±0.4	11.4±0.2
22:5 n-3	2.9±0.4	2.4±0.1	3.2±0.1	2.3±0.2	3.3±0.1	2.2±0.8
22:6 n-3	2.0±0.5	1.9±0.1	1.1±0.1	2.9±0.5	2.9±0.3	3.2±0.2
24:0						
Others						

Example 5

Auxorophic growth

Material and methods

Design of double-gene vector pPhOS2 and transformation cassettes

The EcoRI –HindIII fragment of of pPha –T1 vector containing MCS was replaced by the synthetic sequence comprising of *fcpA* terminator and *fcpA* promoter flanked by 3 multiple cloning sites (MCSs) with unique restriction sites (Fig. 16). The coding sequences for *O. tauri* Δ5-elongase OtElo5 was inserted as *KpnI-SacI* fragment into position 1 of pPhOS vector generating pPhOS2.1.1 construct. The codon optimized for expression in *P.tricornutum* coding sequences for glucose transporters from

Physcomitrella patens (designated Ppglut1), and human erythrocytes (designated Hsglut1), were inserted as *BamHI- XbaI* fragments into position 2 of pPhOS2.1.1 generating pPhOS_Ppglut and pPhOS_HSglut constructs. The resulting constructs were used to transform *P. tricornutum* via biolistics.

Results

Multiple (>10) independent zeocin-resistant colonies were obtained by transformations with these two expression cassettes and used to inoculate cultures for further GC-MS analysis. Transgenic *P. tricornutum* strains expressing pPhOS_Ppglut and pPhOS_HSglut constructs accumulating DPA and elevated levels of DHA were selected for further analysis. (Fig. 16 and Fig. 17). The transformants were transferred to solid medium containing 0.5% of glucose, placed in complete darkness and monitored for growth (Fig. 18).

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Sequence listing

Nucleic acids analogous to cDNA are shown.

SEQ ID No 1 Nucleic acid sequence OtElo5

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taa

SEQ ID No 2 Amino acid sequence OtElo5

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SEQ ID No 3 OtD6 nucleic acid sequence

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SEQ ID No 4 OtD6 amino acid sequence

MCVETENNDGIPTVEIAFDGERERAEANVKLSAEKMEPAALAKTFARRYVVIEGVEYDVT
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SEQ ID No 5 OtD6Pt nucleic acid sequence optimised codon

ggtaccaagcttgatatcaccaaa**atg**tgtgtcgaaacggaaaacaacgatggaatccccacgg
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cagggatgggtcaactggctcatgggttacttgaactgtcaagtcatccaccacctcttcccct
ccatgccgcagtttcgtcaacccgaagtctcgcgtcgcttcgtcgcctttgccaagaagtggaa
cttgaactacaaggtcatgacctacgccggagcctggaaggccacgcttggaaaccttgataac
gtcggaaagcactactacgtccacggccagcactcgggaaagaccgcctaagagctcggtaccc
tcgag

SEQ ID No 6 OtD6 amino acid sequence optimised codon

MCVETENNDGIPTVEIAFDGERERAEANVKLSAEKMEPAALAKTFARRYVVIEGVEYDVT
DFKHPGGTVIFYALSNTGADATEAFKEFHHRSRKARKALAALPSRPAKTAKVDDAEMLQD
FAKWRKELERDGFFKPSPAHVAYRFAELAAMYALGTYLMYARYVVSSVLVYACFFGARCG
WVQHEGGHSSLTGNIWWDKRIQAFTAGFGLAGSGDMWNSMHNKHHATPQKVRHDMDLDTT
PAVAFFNTAVEDNRPRGFSKYWLRLQAWTFIPVTSGLVLLFWMFFLHPSKALKGGKYEEL
VWMLAAHVIRTWTIKAVTGFTAMQSYGLFLATSWVSGCYLFAHFSTSHTHLDVVPADEHL
SWVRYAVDHTIDIDPSQGWVNWLMGYLNCQVIHHLFPSMPQFRQPEVSRRFVAFAKKWNL
NYKVMTYAGAWKATLGNLDNVGKHYYVHGQHSGKTA

SEQ ID No 7 Δ6-desaturase nucleic acid from *Ostreococcus RCC809*

atgcgcgtcgaaacggaggacgacaacgttccgacggtcaccgtcggactgtcggaggag
agcgacgggatgaaggggcgagaaaccccggggcgcgggcgtggaaatcgacgctcgag
ccgcacgcggtggccaagtcgttcgatcgacggtgggtcaaggttgacggcgtcgagtac
gacgtcacggattttaagcatccgggtggatctgtgatttattacatgctgtcgaacacc

ggagcggacgcgacggaggcgttcaaagagtttcattatcggtcgaaaaaggcgagaaag
gcgttggcggcgttgccgcagcgcgagccggaggacgcgtcgccagtggaagacgcgaat
atgttgaaggatttcgcgaaatggcgcaaagatttggagcgcgagggtttctttaaaccg
tcgccggcgcacgtggcgtacagattcgcggaactcgcggccatgttcgcgctcgggacg
gcgttgatgtacgctcgatggcacgccacctcagtcttcgtcaccgcgtgcttttcggc
gcgcggtgcggttgggtgcaacacgagggtggtcacagctcgctgacggggagcatttgg
tgggacaagcgaatccaagcgttcaccgccggtttcggattagcatcgagcggcgacatg
tggaacctcatgcacaacaagcaccacgccactccgcaaaaggtgcgacacgacatggac
ctcgacaccacgccggcggtggccttcttcaacactgcggtcgaggaaaaccgtccgcgc
aagttcagtaagttatggttgcgcgtgcaggcgtggacgttcgtcccggtcacctctggt
ttggtgttgctcgcctggatgtacctcttgcatccgagacacattgctcgccgtaaaaac
tacgaagaggctgcgtggatcgtcgccgcgcacgtcatccgcacgtcggtcatcaaagcc
gtgaccggttactcctggatcacgtgctacggtttgttcttgtccaccatgtgggtgagc
ggctgctacctctttgcgcacttctccacgtctcacacgcacctcgacgtcgttccgagc
gataagcatctctcttgggtgcgatacgccgtcgaccacaccatcgacatcgacccgagc
aagagcgtcgtcaactggttgatgggttacctgaactgccaggtcatccatcacttgttt
ccggacatgcctcagttccgtcagcccgaagtctctcgccgcttcgtctcctttgcgaaa
aagtggaacctcaattacaaggtcatgagctactacggcgcgtggaaggccaccttcggt
aacttgaacgaggtcggcaagcactattacatccaaggttctcaaatcacgaagaagacg
gtgtaa

SEQ ID No 8 Δ6-desaturase amino acid from *Ostreococcus RCC809*

MRVETEDDNVPTVTVGLSEESDGMKGARNPGARAWKSTLEPHAVAKSFDRRWVKVDGVEYDVTD
FKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKALAALPQREPEDASPVEDANMLKDFAKW
RKDLEREGFFKPSPAHVAYRFAELAAMFALGTALMYARWHATSVFVTACFFGARCGWVQHEGGH
SSLTGSIWWDKRIQAFTAGFGLASSGDMWNLMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEE
NRPRKFSKLWLRVQAWTFVPVTSGLVLLAWMYLLHPRHIARRKNYEEAAWIVAAHVIRTSVIKA
VTGYSWITCYGLFLSTMWVSGCYLFAHFSTSHTHLDVVPSDKHLSWVRYAVDHTIDIDPSKSVV
NWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFVSFAKKWNLNYKVMSYYGAWKATFGNLNEVGKH
YYIQGSQITKKTV

SEQ ID No 9 Δ6-desaturase (Ost809Δ6) nucleic acid from *Ostreococcus RCC809* codon optimised for expression in *T.pseudonana*

atgcgtgtggaaaccgaagacgataatgtgccaactgttactgtgggattgtcagaggagtccg
atggaatgaagggagcaaggaaccccggagcacgtgcttggaagtcgacgttggagccgcacgc
cgtggcaaagtcattcgatcgtaggtgggttaaggttgacggagtcgaatacgacgtaactgat
ttcaagcatcccggaggatcagttatctactatatgctttctaacaccggagctgatgccactg
aggctttcaaggaatttcactatcgtagtaagaaggccaggaaggcacttgctgccctcccaca

acgtgagcctgaagacgcttcgccagtcgaggatgccaatatgctcaaggacttcgcaaagtgg
cgtaaggatttggagagggaaggattctttaagccaagtcctgctcacgtggcctaccgtttcg
ccgaactcgcagctatgtttgctttgggaactgcccttatgtatgcacgttggcatgctacgtc
tgtcttcgtaacagcctgtttctttggagcaaggtgtggatgggtgcaacacgagggaggacat
tcttccttgaccggatccatctggtgggataagcgtattcaggcattcactgctggatttggac
ttgccagttcgggagacatgtggaacctcatgcacaataagcaccatgcaacgccacaaaaagt
taggcatgatatggacctcgataccactcctgcagtggctttctttaacacagctgttgaggaa
aatcgtcctaggaagttctctaagttgtggcttcgtgtccaggcctggacctttgtgcccgtta
cttccggattggtactcttggcatggatgtaccttctccacccgcgtcatatcgctcgtaggaa
gaactatgaggaagccgcatggattgtggctgcccatgttatcaggacctccgtcattaaggct
gtaacgggatacagttggatcacatgttatggactcttcttgtcgactatgtgggtctcaggat
gctacctcttcgctcacttttcaacgtctcacacacatttggacgtggttccatctgataagca
cctttcctgggtgcgttacgccgttgatcataccatcgacattgatccttccaagagtgtcgta
aactggctcatgggatatttgaactgtcaggttatccaccatttgttccccgacatgccgcaat
ttcgtcagcccgaagtcagtcgtaggttcgtatcgtttgccaagaagtggaaccttaattacaa
ggtcatgtcttactatggagcctggaaggcaaccttcggaaatctcaacgaagtcggaaagcac
tactacatccaaggaagtcaaatcacaaagaagacggtt**tag**

SEQ ID No 10 Δ6-desaturase amino acid from *Ostreococcus RCC809* codon optimised

MRVETEDDNVPTVTVGLSEESDGMKGARNPGARAWKSTLEPHAVAKSFDRRWVKVDGVEY
DVTDFKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKALAALPQREPEDASPVEDAN
MLKDFAKWRKDLEREGFFKPSPAHVAYRFAELAAMFALGTALMYARWHATSVFVTACFFG
ARCGWVQHEGGHSSLTGSIWWDKRIQAFTAGFGLASSGDMWNLMHNKHHATPQKVRHDMD
LDTTPAVAFFNTAVEENRPRKFSKLWLRVQAWTFVPVTSGLVLLAWMYLLHPRHIARRKN
YEEAAWIVAAHVIRTSVIKAVTGYSWITCYGLFLSTMWVSGCYLFAHFSTSHTHLDVVPS
DKHLSWVRYAVDHTIDIDPSKSVVNWLMGYLNCQVIHHLFPDMPQFRQPEVSRRFVSFAK
KWNLNYKVMSYYGAWKATFGNLNEVGKHYYIQGSQITKKTV

SEQ No. 11 Δ4-desaturase from *E. huxleyi* (EhD4) codon-optimized for expression in Arabidopsis

atgggaggcgccggcgcgagcgaggctgaacggcccaagtggaccacgatccacgggcggcacg
tcgatgtgtcaaagttccgccacccgggtgggaacatcatcgagctcttctatggcatggactc
gacgagcgcgttcgagcagttccacggccaccacaagggcgcgtggaagatgctcaaggcgctg
ccgaccaaggaggtcgaccccgccgacgtgccgcagcagccgcaggagcacgttgccgagatga
cgcggctgatgacgtcgtggcgcgagcgcggcctctttaagccgcgccccgtcgcctcgggcat
ctacggtctcgccgtcgtcgctgccatcgtcgcgtgcatcgcctgcgcgccgcacgcgccggtg
ctgagcgggatcgggctcggcagctgctgggcgcagtgcggcttcctgcagcacatgggcgggc
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cgggtccgcctcgtggtggcgcaaccgccacaacaagcatcacgcaaagactaacgtgctcggc
gaggacggcgacctgcggacgactcccttcttcgcctgggacccgacgctcgccaagaaggttc
cagactggtcgctcaagacgcaggccttcaccttcctccccgccctcggagcgtacgtctttgt
ctttgccttcacgatccgcaagtatgccgtcgtcaagaagctctggcacgagctcgcactcatg
atcgcgcactacgcgatgttctactacgcgctgcagctcgccggtgcgtcgctcggcagcggcc

tcgcctttactgcaccggctacgcctggcaaggcatctacctcggcttcttcttcggcctgtc
ccacttcgcggtcgagcgagtcccctccaccgccacctggctcgagtcgtccatgatcggcacc
gtcgactggggaggctcctccgcctttgcggctacgtctccggcttcctcaacatccagatcg
agcaccacatggcgccgcagatgccgatggagaacctgcgccagatccgcgccgactgcaaggc
gagcgcggagaagctcgggcttccctatcgcgagctctccttcgccggcgcggtcaagctgatg
atggtcggcctctggcgcacggggagggacgagctgcagctgcgctccgacaggcgcaagtact
cgcgcacccaggcctacatggcggccgcctcggcggtggtggagaacctcaaggcggactag

SEQ No. 12 Δ4-desaturases from *E. huxleyi* codon-optimized for expression in Arabidopsis

MGNGNLPASTAQLKSTSKPQQQHEHRTISKSELAQHNTPKSAWCAVHSTPATDPSHSNNKQHAH
LVLDITDFASRHPGGDLILLASGKDASVLFETYHPRGVPTSLIQKLQIGVMEEEAFRDSFYSWT
DSDFYTVLKRRVVERLEERGLDRRGSKEIWIKALFLLVGFWYCLYKMYTTSDIDQYGIAIAYSI
GMGTFAAFIGTCIQHDGNHGAFAQNKLLNKLAGWTLDMIGASAFTWELQHMLGHHPYTNVLDGV
EEERKERGEDVALEEKDQESDPDVFSSFPLMRMHPHHTTSWYHKYQHLYAPPLFALMTLAKVFQ
QDFEVATSGRLYHIDANVRYGSVWNVMRFWAMKVITMGYMMGLPIYFHGVLRGVGLFVIGHLAC
GELLATMFIVNHVIEGVSYGTKDLVGGASHGDEKKIVKPTTVLGDTPMEKTREEALKSNSNNNK
KKGEKNSVPSVPFNDWAAVQCQTSVNWSPGSWFWNHFSGGLSHQIEHHLFPSICHTNYCHIQDV
VESTCAEYGVPYQSESNLFVAYGKMISHLKFLGKAKCE*

SEQ ID No. 13 D4-desaturase from *Thalassiosira pseudonana* nucleic acid

atgggcaacggcaacctcccagcatccaccgcacagctcaagtccacctcgaagccccagcagc
aacatgagcatcgcaccatctccaagtccgagctcgcccaacacaacacgcccaaatcagcatg
gtgtgccgtccactccactcccgccaccgacccatcccactccaacaacaaacaacacgcacac
ctagtcctcgacattaccgactttgcgtcccgccatccaggggagacctcatcctcctcgctt
ccggcaaagacgcctcggtgctgtttgaaacataccatccacgtggagttccgacgtctctcat
tcaaaagctgcagattggagtgatggaggaggaggcgtttcgggattcgttttacagttggact
gattctgacttttatactgtgttgaagaggagggttgtggagcggttggaggagaggggttgg
acaggaggggatcgaaagagatttggatcaaggctttgttcttgttggttggattttggtactg
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ggaatgggaacctttgcggcattcatcggcacgtgtattcaacacgatggaaatcacggtgcat
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gtttacgtgggagcttcagcacatgctggggcatcatccatatacgaatgtgttggatggggtg
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cagacgtattctcctccttccctctcatgagaatgcatccccaccatacaacctcatggtatca
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caggattttgaagttgccacatccggacgattatatcatattgatgccaatgtacgttatggtt
cggtatggaatgtcatgaggttttgggctatgaaggtcattacgatgggatatatgatgggatt
accaatctactttcatggagtactgaggggagttggattgtttgttattgggcatttggcgtgt
ggagagttgttggcgacgatgtttattgtgaatcacgtcattgagggtgtgagttatggaacga

aggatttggttggtggtgcgagtcatggagatgagaagaagattgtcaagccaacgactgtatt
gggagatacaccaatggaaaagactcgcgaggaggcattgaaaagcaacagcaataacaacaag
aagaagggagagaagaactcggtaccatccgttccattcaacgactgggcagcagtccaatgcc
agacctccgtgaattggtctccaggctcatggttctggaatcacttttctgggggactctctca
tcagattgagcatcacttgttccccagcatttgtcatacaaactactgtcatatccaggatgtt
gtggagagtacgtgtgctgagtacggagttccgtatcagagtgagagtaatttgtttgttgctt
atggaaagatgattagtcatttgaagtttttgggtaaagccaagtgtgagtag

SEQ ID No. 14 D4-desaturase from *Thalassiosira pseudonana* amino acid acid

MGGAGASEAERPKWTTIHGRHVDVSKFRHPGGNIIELFYGMDSTSAFEQFHGHHKGAWKM
LKALPTKEVDPADVPQQPQEHVAEMTRLMTSWRERGLFKPRPVASGIYGLAVVAAIVACI
ACAPHAPVLSGIGLGSCWAQCGFLQHMGGHREWGVRYSFLLQHFFEGLLKGGSASWWRNR
HNKHHAKTNVLGEDGDLRTTPFFAWDPTLAKKVPDWSLKTQAFTFLPALGAYVFVFAFTI
RKYAVVKKLWHELALMIAHYAMFYYALQLAGASLGSGLAFYCTGYAWQGIYLGFFFGLSH
FAVERVPSTATWLESSMIGTVDWGGSSAFCGYVSGFLNIQIEHHMAPQMPMENLRQIRAD
CKASAEKLGLPYRELSFAGAVKLMMVGLWRTGRDELQLRSDRRKYSRTQAYMAAASAVVE
NLKAD*

SEQ ID No. 15 Δ4-desaturase *Ostreococcus* RCC809 nucleic acid

atgccgacgactcgatcgcgcgcgcgtgacgacgccccctcgcgagacgccgacgagagcga
acaccgtcgccgcgctcgatcccgagcgcaagtacacgcgcattcgcggcgtcgtgtacgacgt
cacggatttcgccagccgtcatccgggtggcgcgcaattgttatcgctgtgcgtggggagagac
gccaccatcctggtggagagtcatcaccttcgtccggaggtggtgcaaaagtacctgaagacgc
ttcccgtggtggagggcgcggcgggggcgttcgggcccgaggagacgtttccgaaaccgctcga
ctcggatttgtaccgaaagattcaggggcgcgttcgtaaagagatcgtcgaaccgttgaagatg
acgcgcggacgcgagccgcacgggcgaggctggtgcgtgttggacgccggggtggtgttggctt
tcttcgcgttcgcgttgggagtctattggaagacgccgacggtggcgacggggtgcctgttggg
gctcgccgggtactggagcggcaccggattgcaacacacggcgaaccacggtggattggcgaag
agtgggttttggaatcagttttggggatggctcgggaacgacgtcgccatcgggaagagctcgg
tggagtggagatatcatcacatggtgagccaccactcgtattgcaacgacgcggacctcgatca
agacgtgtacaccgcgctgccgcttcttcgtttggacccgtcccaggagttgaagtggttccac
cgctaccaagcgttctacgcgccgctgatgtggccgatgttgtggctcgccgcgcagtttggcg
acgcgcaaaatattttagtggataaggcgtctccgggcgtcgagtacaagggcctcatgaagct
cgaagtcgcgctgtacgttctcggaaagtttttgcattttagcttgttgctcggcgtaccggcc
tacttgcacgggtttgcgaacgccatcgtgccgttcatcgcgtacggtgcgttcggttcgttcg

tcctgtgctggtttttcatcgtcagtcacaacttggaggcgttgaccccaatcaatctgagcaa
atccacgaagaatgactggggcgcgtggcaaatcgaaacttccgcgtcctggggcaacggcttc
tggagcttttctccggcgggttgaatttgcaaatcgagcaccacttgttcccgggttgcgcgc
acaacttgtacccgaagatggttcccatcatcaaggaagagtgcgaaaaggctggcgtcacgta
caccggttacggtgggtactttggtctccttcccatcactcgggacatgttcgcgtacttgtac
aaaatgggccgacaaagcaaaaagtcggcgtaa

SEQ ID No. 16 Δ4-desaturase *Ostreococcus* RCC809 amino acid

MPTTRSRARVTTPPRETPTRANTVAALDPERKYTRIRGVVYDVTDFASRHPGGAQLLSLCVGRD
ATILVESHHLRPEVVQKYLKTLPVVEGAAGAFGPEETFPKPLDSDLYRKIQGRVRKEIVEPLKM
TRGREPHGRGWCVLDAGVVLAFFAFALGVYWKTPTVATGCLLGLAGYWSGTGLQHTANHGGLAK
SGFWNQFWGWLGNDVAIGKSSVEWRYHHMVSHHSYCNDADLDQDVYTALPLLRLDPSQELKWFH
RYQAFYAPLMWPMLWLAAQFGDAQNILVDKASPGVEYKGLMKLEVALYVLGKFLHFSLLLGVPA
YLHGFANAIVPFIAYGAFGSFVLCWFFIVSHNLEALTPINLSKSTKNDWGAWQIETSASWGNGF
WSFFSGGLNLQIEHHLFPGCAHNLYPKMVPIIKEECEKAGVTYTGYGGYFGLLPITRDMFAYLY
KMGRQSKKSA*

SEQ ID No. 17 Δ4-desaturase *Ostreococcus* RCC809 nucleic acid codon optimised acid for expression in Pt

ggatccggtaccaagcttgatatcaccaaa**atg**ccaactactcgttctcgtgctcgtgttacta
ctccacctcgtgaaactcctactcgtgctaatactgttgctgctttagatccagaacgtaaata
tacacgtattcgaggtgttgtatatgatgttactgattttgctagtcgacatccaggtggtgca
caattattatctttatgtgttggtcgtgatgctacaattttagtagaatcacatcatttacgac
cagaagttgtacaaaaatatttaaaaacattacctgttgtagaaggtgctgctggtgcatttgg
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cgaaaagaaattgtagaaccattaaaaatgacacgtggtcgagaacctcatggtcgtggttggt
gtgttttagatgctggtgttgtattagctttctttgcttttgcattaggtgtttattggaaaac
accaactgtagctactggttgtttattaggtttagcaggttattggtctggtacaggtttacaa
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agttattgtaatgatgctgatttagatcaagatgtttatacagcattaccattattacgtttag
atccttcacaagaattaaaatggtttcatcgttatcaagcattttatgcacctttaatgtggcc
tatgttatggttagctgcacaatttggtgatgctcaaaatattttagttgataaagcaagtcca
ggtgtagaatataaaggtttaatgaaattagaagttgctttatatgtattaggaaaatttttta

cattttctttattattaggtgttcctgcatatttacatggttttgctaatgcaattgtaccat
ttattgcttatggtgcatttggttcatttgttttatgttggtttttcattgtaagtcataattt
agaagcattaacaccaattaatttatctaaatcaactaaaaatgattggggtgcttggcaaatt
gaaactagtgcatcttggggtaatggtttttggtcatttttctcaggtggtttaaatttacaaa
ttgaacatcatttatttcctggttgtgctcataatttatatccaaaaatggttcctattattaa
agaagaatgtgaaaaagcaggtgttacatatactggttatggtggttattttggtttattacca
attactcgtgatatgtttgcttatttatataaaatgggtcgtcaatctaaaaaatctgcttaag
agctcggtaccctcgagtctaga

SEQ ID No. 18 Δ4-desaturase *Ostreococcus* RCC809 amino acid codon optimised acid for expression in Pt

MPTTRSRARVTTPPRETPTRANTVAALDPERKYTRIRGVVYDVTDFASRHPGGAQLLSLCVGRD
ATILVESHHLRPEVVQKYLKTLPVVEGAAGAFGPEETFPKPLDSDLYRKIQGRVRKEIVEPLKM
TRGREPHGRGWCVLDAGVVLAFFAFALGVYWKTPTVATGCLLGLAGYWSGTGLQHTANHGGLAK
SGFWNQFWGWLGNDVAIGKSSVEWRYHHMVSHHSYCNDADLDQDVYTALPLLRLDPSQELKWFH
RYQAFYAPLMWPMLWLAAQFGDAQNILVDKASPGVEYKGLMKLEVALYVLGKFLHFSLLLGVPA
YLHGFANAIVPFIAYGAFGSFVLCWFFIVSHNLEALTPINLSKSTKNDWGAWQIETSASWGNGF
WSFFSGGLNLQIEHHLFPGCAHNLYPKMVPIIKEECEKAGVTYTGYGGYFGLLPITRDMFAYLY
KMGRQSKKSA*

SEQ ID No. 19 Δ6-elongase from *Fragilariopsis cylindrus* nucleic acid

ccatggggtaccgatatcaccaaa**atg**gacgagtacaaagcaactcttgaatctgt
tggggatgctatcatccaatgggcagatcctgaaagtcagttcaccgggttcacca
agggatggttcttgacagatttcacatctgcgtttagtattgcacttgtatacgtc
ttatttgtcatcattggttctcaagtgatgaaagtcttacctgctattgatccgta
cccaatcaagtttttttacaatgtatcacaaattatgctgtgtgcttacatgacga
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gcgaatgtcttttatgatggtgatatttatcttaccattgctctgaatggtttcat
ccatactgttatgtacacatactactttatctgtatgcatactaaagacaagaaaa
ctggaaaatcgcttcctatctggtggaaatcatctttgactttgttgcaattgttt
cagttcattaccatgatgtcacagggcttataccttatcatttttggttgtgaatc

actttctatccgagtcactgcgacatacgttgtttacatattgtcacttttctttt
tgtttgcgcaattcttcgttgcatcttacatgcaacctaagaaatcgaagactgcc
taagagctcggtaccttaattaa

SEQ ID No. 20 Δ6-elongase from *Fragilariopsis cylindrus* amino acid

MDEYKATLESVGDAIIQWADPESQFTGFTKGWFLTDFTSAFSIALVYVLFVIIGSQVMKVLPAI
DPYPIKFFYNVSQIMLCAYMTIEACLLAYRNGYTIMPCVGYNRDDPAIGNLLWLFYVSKVWDFW
DTIFIVLGKKWRQLSFLHVYHHTTIFLFYWLNANVFYDGDIYLTIALNGFIHTVMYTYYFICMH
TKDKKTGKSLPIWWKSSLTLLQLFQFITMMSQGLYLIIFGCESLSIRVTATYVVYILSLFFLFA
QFFVASYMQPKKSKTA

SEQ ID No. 21 Δ5-desurase from *Fragilariopsis cylindrus* nucleic acid

```
   1 ATGGCACCCGACGCCGATCACAAGCTGAGACAGCGCCGTCTAAAAGGCGACGAAGTTTGT
  61 ATCGATGGAATTATCTATGATATATCATCCTTCGAGCATCCGGGTGGTGATACTATCAAC
 121 GTATTTGGTGGAAACGATGCAACAATTCAGTACAAAATGATTCACCCGTACCATACCACG
 181 AAGCATTTAGAAAAAATGAAGGTAGTTGGTAAAGTTCCAGACTACTACTCAGAATACAAA
 241 TGGGATACACCCTTCGAACGTGAAATGAAACGTGAGGTATTTAAAATTGTACGACGTGGA
 301 CAAGAATTTGGTACAAATGGATATTTTTTCCGTGCCATTTCGTATATTGCTATGTTTTTT
 361 TATCTGCAATATTTATGGATGCAAGAATCTTCCTACACGTTAGCCATCGTATACGGGATT
 421 AGTATGGGATTGATTGGACTGAATGTCCAGCATGATGCGAACCACGGAGCTGCATCGAAA
 481 AAAGTGTGGGTGAATGACCTCCTAGGATTGGGAGCAGACTTTATCGGAGGATCGAAATGG
 541 TTGTGGATGGAAAAACATTGGACGCATCATGCTTTTACAAACCATCGAGAAAAGGATCCA
 601 GATGGGTTAGCAGCGGAACCTTTCCTATTGTTCAACGACTACGACTTGTCGAGTTCCAAA
 661 CGTGCTGGATATCATGCATACCAAGGAATTTATTTAGTCCTATTATTGTGTGGGTATTGG
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1141 AGAATGTCTAGTGCTTGGTATCCATTTATTGCACCAAAAGTTCGTGAAATTTGCAAAAAG
1201 CACGGAGTTCGTTACGTATACTATCCATGGTTGTTGCAAAATATGTATTCGACGTTGAAG
1261 TACACCCACGAGGTTGGTGTCGGCTCACATTGGAAGGATAATCCTTTTAAGGGTGAAATG
1321 TAG
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SEQ ID No. 22 Δ5-desurase from *Fragilariopsis cylindrus* amino acid

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  61  KHLEKMKVVGKVPDYYSEYKWDTPFEREMKREVFKIVRRGQEFGTNGYFFRAISYIAMFF
 121  YLQYLWMQESSYTLAIVYGISMGLIGLNVQHDANHGAASKKVWVNDLLGLGADFIGGSKW
 181  LWMEKHWTHHAFTNHREKDPDGLAAEPFLLFNDYDLSSSKRAGYHAYQGIYLVLLLCGYW
 241  LSAIIDIPVIWNLQDRGALTVGIQLDNDWIASRRKYAVSLRILYLFCNIVVPLYNNFSWT
 301  TVSHINVMGICGSLTLGLLFTLSHNFENVDRDPTNLNLNETEEPVCWFKSQVETSSTYGG
 361  MISGWLTGGLNFQVEHHLFPRMSSAWYPFIAPKVREICKKHGVRYVYYPWLLQNMYSTLK
 421  YTHEVGVGSHWKDNPFKGEM-
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SEQ ID No. 23 P. patens PpHUP1L codon-optimised for expression in *Phaeodactylum tricornutum*

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    361
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    901
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    961
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   1021
GTTGTGGATCGATTCGGTCGACGATGGTTGCTCTTGGAAGCTTGCATCCAAATGTTCTTA
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1261
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1501
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1561 TCCGCAAATGGCCACAAACTGTAA

SEQ ID No. 24 Deduced polypeptide sequence of **PpHUP1L**

1 MAGGGVVTAGEIKHYPGRTTFFVIMVCIVAASGGLMFGYDVGISGGVTSMDEFLAKFFPA
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181
IGILFASLVNYGTEKMARNGWRVSLAIAGLPAIFITLGGLLLPDTPNSLVQRGKHESARQ
241
VLRRIRGVDNIEEEFDDILIASNEAASVKHPFRNILKRRNRPQLVISMALQFFQQFTGIN
301
AIMFYAPVLFQTLGFGSSASLYSAVIVGAVNVLATCVAIAVVDRFGRRWLLLEACIQMFL
361
AQTAIAIILAAGLKGTEMPEYLGWIAVVLICVYVSSFAWSWGPLGWLIPSEIFPLETRSA
421
GQAITVSTNMVFTFLIAQVFLSMLCAFKWGIFLFFAAWVVVMFLFTYFLIPETKGIPIEE
481 MDLVWTKHWFWKRYVPYPETLAHTSGIPMGDMKVSKLENGSANGHKL-

SEQ ID No. 25 Homo sapiens HsGLUT1 codon-optimised for expression in *Phaeodactylum tricornutum*

1
ATGGAGCCCAGCAGCAAGAAGCTGACGGGTCGCCTCATGCTGGCTGTGGGAGGAGCAGTG
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121
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CTCACCACGCTCTGGTCCCTCTCAGTGGCCATCTTTTCTGTTGGGGGCATGATTGGCTCC

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TTCTCTGTGGGCCTTTTCGTTAACCGCTTTGGCCGGCGGAATTCAATGCTGATGATGAAC
301
CTGCTGGCCTTCGTGTCCGCCGTGCTCATGGGCTTCTCGAAACTGGGCAAGTCCTTTGAG
361
ATGCTGATCCTGGGCCGCTTCATCATCGGTGTGTACTGCGGCCTGACCACAGGCTTCGTG
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CAGCTGGGCATCGTCGTCGGCATCCTCATCGCCCAGGTGTTCGGCCTGGACTCCATCATG
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GGCAACAAGGACCTGTGGCCCCTGCTGCTGAGCATCATCTTCATCCCGGCCCTGCTGCAG
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TGCATCGTGCTGCCCTTCTGCCCCGAGAGTCCCCGCTTCCTGCTCATCAACCGCAACGAG
661
GAGAACCGGGCCAAGAGTGTGCTAAAGAAGCTGCGCGGGACAGCTGACGTGACCCATGAC
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781
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841
TCCCAGCAGCTGTCTGGCATCAACGCTGTCTTCTATTACTCCACGAGCATCTTCGAGAAG
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1141
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1201
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1381
GATGAGATCGCTTCCGGCTTCCGGCAGGGGGGAGCCAGCCAAAGTGATAAGACACCCGAG
1441 GAGCTGTTCCATCCCCTGGGGGCTGATTCCCAAGTGTGA

SEQ ID No. 26 **Deduced polypeptide sequence of HsGLUT1**

1
MEPSSKKLTGRLMLAVGGAVLGSLQFGYNTGVINAPQKVIEEFYNQTWVHRYGESILPTT
61
LTTLWSLSVAIFSVGGMIGSFSVGLFVNRFGRRNSMLMMNLLAFVSAVLMGFSKLGKSFE
121
MLILGRFIIGVYCGLTTGFVPMYVGEVSPTAFRGALGTLHQLGIVVGILIAQVFGLDSIM
181
GNKDLWPLLLSIIFIPALLQCIVLPFCPESPRFLLINRNEENRAKSVLKKLRGTADVTHD
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LQEMKEESRQMMREKKVTILELFRSPAYRQPILIAVVLQLSQQLSGINAVFYYSTSIFEK

301
AGVQQPVYATIGSGIVNTAFTVVSLFVVERAGRRTLHLIGLAGMAGCAILMTIALALLEQ
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LPWMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRPAAIAVAGFSNWTSNFIVGM
421
CFQYVEQLCGPYVFIIFTVLLVLFFIFTYFKVPETKGRTFDEIASGFRQGGASQSDKTPE
481 ELFHPLGADSQV-

CLAIMS:

1. A transgenic microalgae with increased production of omega-3 LC-PUFAs.
2. A transgenic microalgae according to claim 1 wherein the microalgae is a diatom.
3. A transgenic microalgae according to claim 2 wherein the diatom is *P. tricornutum*.
4. A transgenic microalgae according to a preceding claim wherein the omega-3 LC-PUFA is selected from EPA or DHA.
5. A transgenic microalgae according to claim 4 wherein the omega-3 LC-PUFA is DHA.
6. A transgenic microalgae according to claim 5 wherein the increased DHA content as a percentage of total fatty acids is increased by at least 1%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15% or more compared to a control microalgae.
7. A transgenic microalgae according to a preceding claim expressing a nucleic acid encoding a Δ5-elongase.
8. A transgenic microalgae according to claim 7 wherein said nucleic acid comprises SEQ ID No. 1 or a sequence that encodes for a Δ5-elongase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 2.
9. A transgenic microalgae according to any of claims 7 to 9 wherein the transgenic microalgae further comprises one or more nucleic acids encoding for a polypeptide involved in regulation of the LC-PUFA pathway.
10. A transgenic microalgae according to claim 9 wherein said nucleic acid encodes a Δ6-desaturase comprising SEQ ID No. 4 or 6 or a sequence that encodes for a Δ6-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 4 or 6.
11. A transgenic microalgae according to claim 9 wherein said nucleic acid encodes a 6Δ-desaturase comprising SEQ ID No. 8 or 10 or a sequence that encodes for a 6Δ-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 8 or 10.

12. A transgenic microalgae according to any of claims 1 to 4 wherein the omega 3-fatty acid is EPA.
13. A transgenic microalgae according to claim 12 wherein said microalgae expresses a nucleic acid that encodes a Δ6-desaturase.
14. A transgenic microalgae according to claim 12 wherein said nucleic acid encodes a Δ-6desaturase comprising SEQ ID No. 4 or 6 or a sequence that encodes for a Δ6-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 4 or 6.
15. A transgenic microalgae according to claim 12 wherein said nucleic acid encodes a Δ6-desaturase comprising SEQ ID No. 8 or 10 or a sequence that encodes for a Δ6-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology to SEQ ID No. 8 or 10.
16. A transgenic microalgae according to any of claims 7 to 15 wherein said nucleic acid further comprises a regulatory sequence.
17. The use of a transgenic microalgae according to a preceding claim in producing omega-3 LC-PUFAs or increasing one or more omega-3 LC-PUFAs.
18. The use according to claim 17 wherein the omega-3 LC-PUFA is EPA or DHA.
19. The use according to claim 17 wherein the omega-3 LC-PUFAs is DHA.
20. A method for producing transgenic microalgae with increased omega-3 LC-PUFAs content.
21. A method according to claim 20 wherein the omega-3 LC-PUFA is DHA and the method comprises transforming a microalgae with a nucleic acid encoding a Δ5-elongase.
22. A method according to claim 20 wherein the omega-3 LC-PUFA is EPA and the method comprises transforming a microalgae with a nucleic acid encoding a Δ6-desaturase.
23. A method for increasing production of one of more omega-3 LC-PUFA in microalgae comprising

a) cultivating a transgenic microalgae according to any of claims 1 to 16 under conditions which allow for the production of one of more omega-3 LC-PUFAs and

b) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

24. A method according to claim 23 wherein said omega-3 LC-PUFA is DHA and the method comprises

a) cultivating a transgenic microalgae according to any of claims 5 to 11 or 16 under conditions which allow for the production of DHA and

b) obtaining said DHA from the transgenic microalgae.

25. A method according to claim 20 wherein said omega- 3 LC-PUFA is EPA and the method comprises

a) cultivating a transgenic microalgae according to any of claims 12 to 16 under conditions which allow for the production of EPA and

b) obtaining said EPA from the transgenic microalgae.

26. An oil isolated from a microalgae according to any of claims 1 to 16 or a foodstuff, feedstuff, nutriceutical or cosmetic obtained from a microalgae according to any of claims 1 to 16.

27. A composition comprising a transgenic microalgae according to any of claims 1 to 16 or an oil according to claim 26.

28. A composition comprising a transgenic microalgae according to any of claims 1 to 16 for use as a medicament.

29. A composition comprising a transgenic microalgae according to any of claims 1 to 16 for use in the treatment or prevention of cardiovascular conditions, including atherosclerosis, thrombosis, high blood pressure, myocardial infarction and atherosclerosis, inflammatory conditions, depression, cognitive decline, arthritis, eczema, metabolic syndrome and type II diabetes.

30. A transgenic microalgae according to any of claims 1 to 16 or a composition comprising a transgenic microalgae according to any of claims 1 to 16 for use as a foodstuff, feedstuff, nutriceutical or cosmetic.

31. A method for making a feedstuff comprising a) cultivating a transgenic microalgae comprising a heterologous transgene as defined in any of claims 1-16 under conditions which allow for the production of one of more omega-3 LC-PUFAs and

b) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

32. An isolated nucleic acid comprising SEQ ID No. 7 or 9 encoding Δ6-desaturase (Ost809Δ6) comprising SEQ ID No. 8 or 10, a functional variant thereof or a Δ6-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at

least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 8 or 11.

33. An isolated nucleic acid comprising SEQ ID No. 15 or 17 encoding a Δ4-desaturase (Ost809Δ4) comprising SEQ ID No.16 or 18, a functional variant thereof or a Δ4-desaturase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 16 or 18.

34. An isolated nucleic acid comprising SEQ ID No. 19 encoding Δ6-elongase (FcELO6) comprising ID No. 20 a functional variant thereof or a Δ6-elongase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 20.

35. An isolated nucleic acid comprising SEQ ID No. 21 encoding Δ5-desaturase comprising ID No. 22 a functional variant thereof or a Δ6-elongase that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% homology to SEQ ID No. 22.

36. A vector comprising an isolated nucleic according to claim 32, 33, 34 and/or 35.

37. A host cell comprising a vector according to claim 36.

38. A host cell according to claim 37 wherein the host cell is an algae or higher plant cell.

39. The use of an isolated nucleic according to claim 32, 33, 34 and/or 34 in increasing the production of omega-3 LC-PUFAs in microalgae, the preparation of a foodstuff, feedstuff, nutriceutical, cosmetic or medicament.

40. The use according to claim 39 wherein the omega-3 LC-PUFAs is EPA or DHA.

41. A method for increasing production of one of more omega-3 LC-PUFA in microalgae comprising

a) cultivating a transgenic microalgae comprising a heterologous transgene comprising one or more of the nucleic acids defined in claims 30, 31 or 32 under conditions which allow for the production of one of more omega-3 LC-PUFAs and

b) obtaining said one of more omega-3 LC-PUFA from the transgenic microalgae.

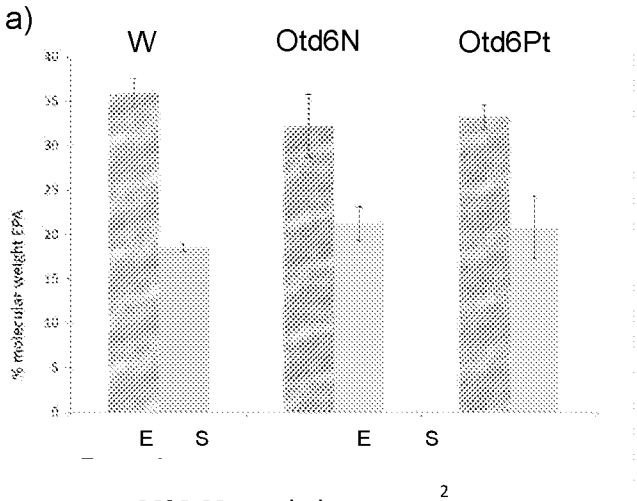
42. A transgenic organism with increased DHA levels expressing a heterologous Δ5-elongase.

43. A transgenic organism according to claim 42 wherein the Δ5-elongase is a Δ5-elongase from *Ostreococcus tauri.*

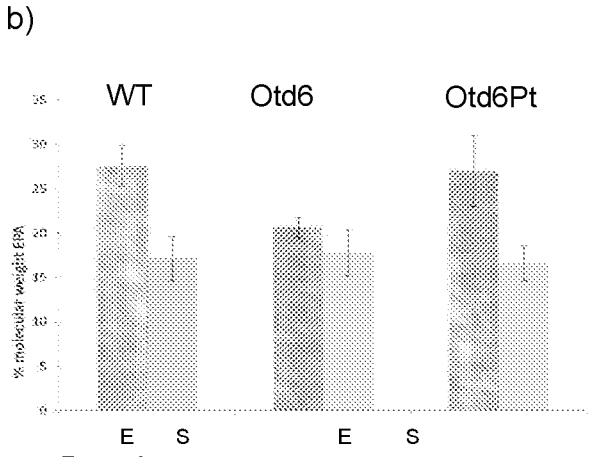
44. A transgenic organism according to claim 42 and 43 wherein no other heterologous transgenes involved in the regulation of the LC-PUFAs biosynthetic pathway are expressed in said organism.

5

FIGURE 1



20°C 60 µmol photons m^{-2} s^{-}



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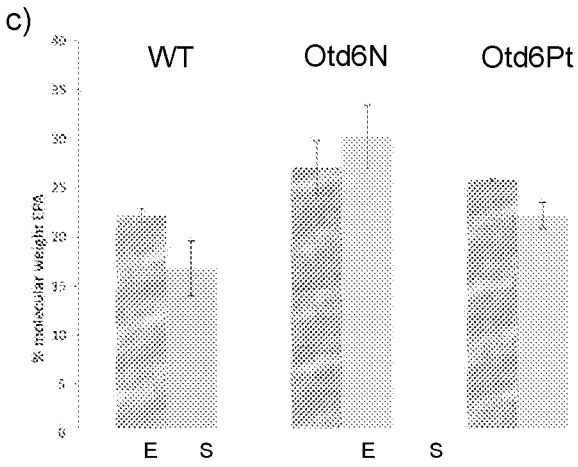


18°C 25 µmol photons m^{-2} s^{-1}

FIGURE 2a

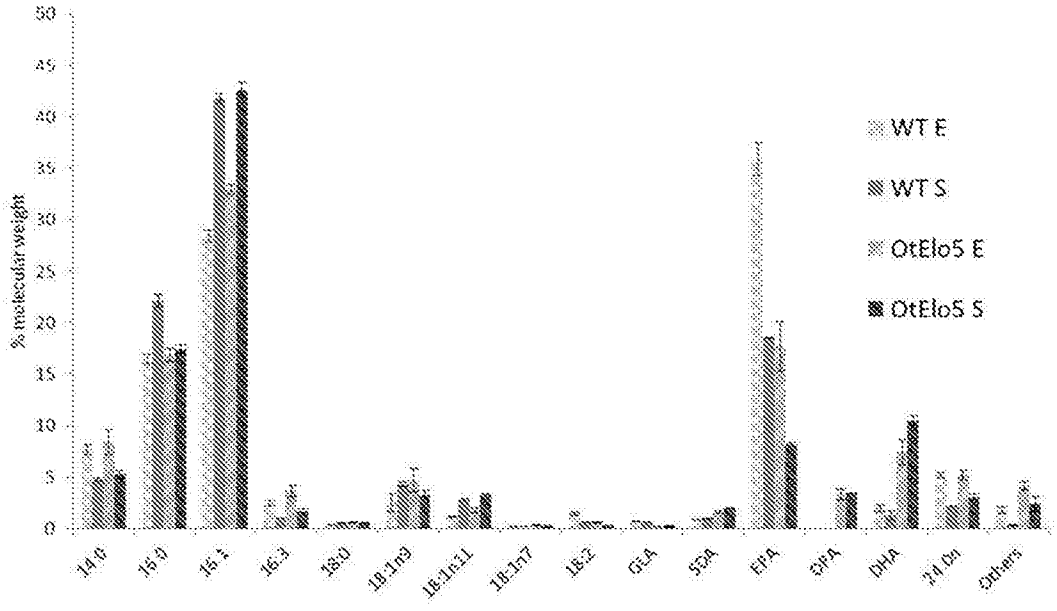


FIGURE 2b

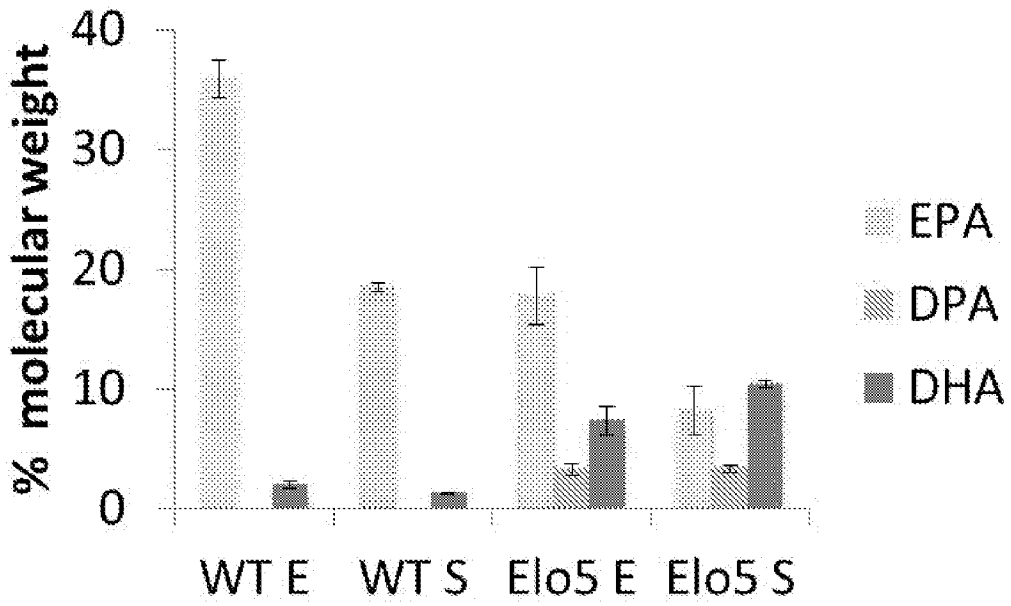


FIGURE 3a

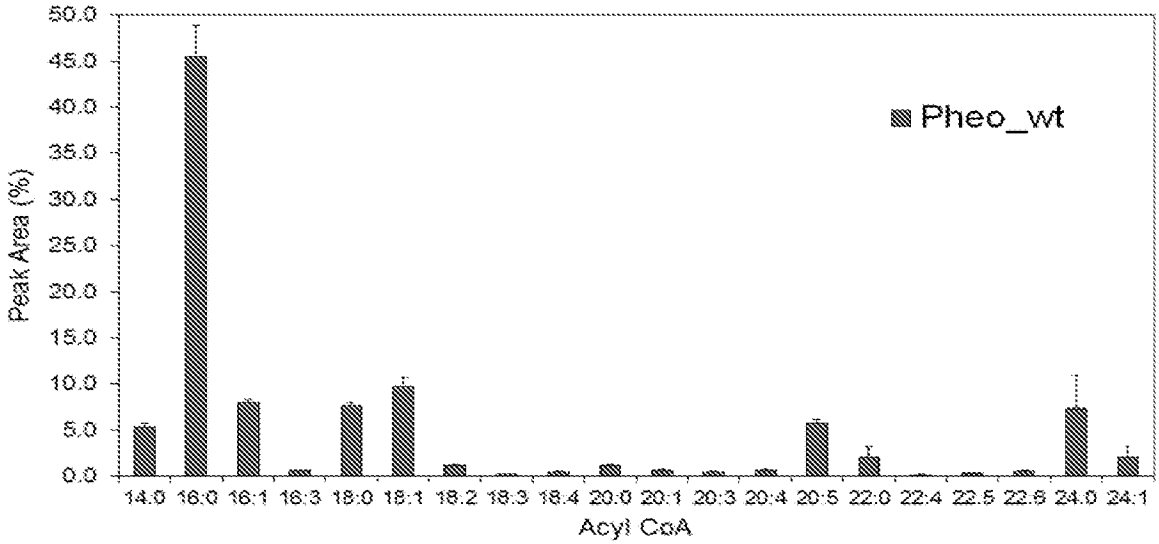


FIGURE 3b

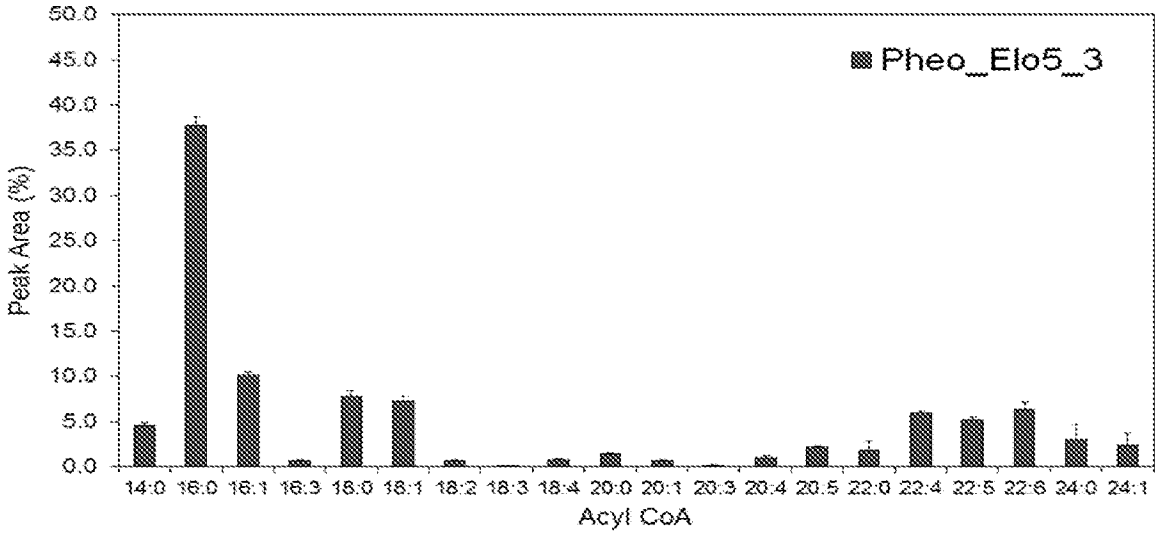


FIGURE 4a

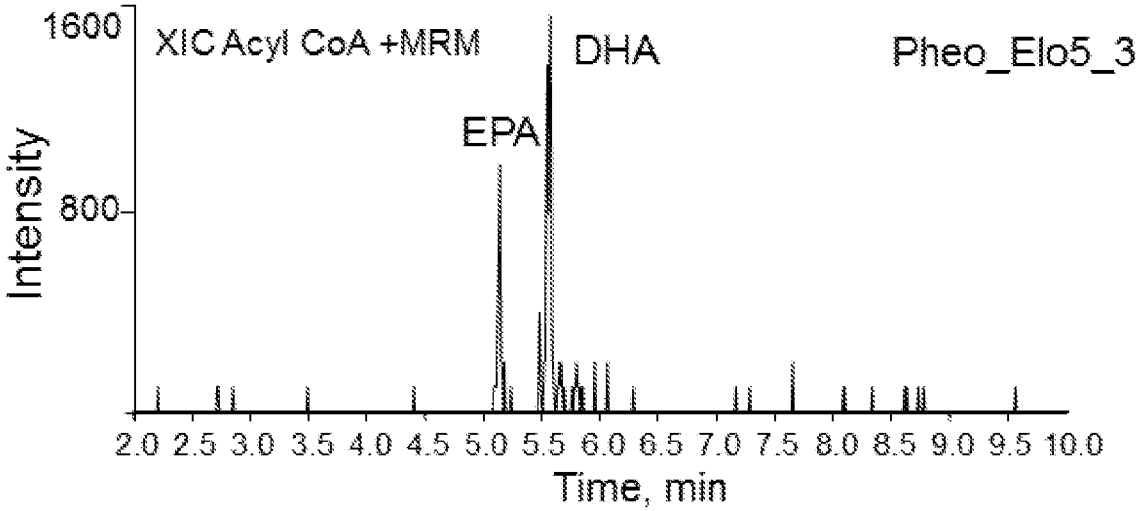


FIGURE 4b

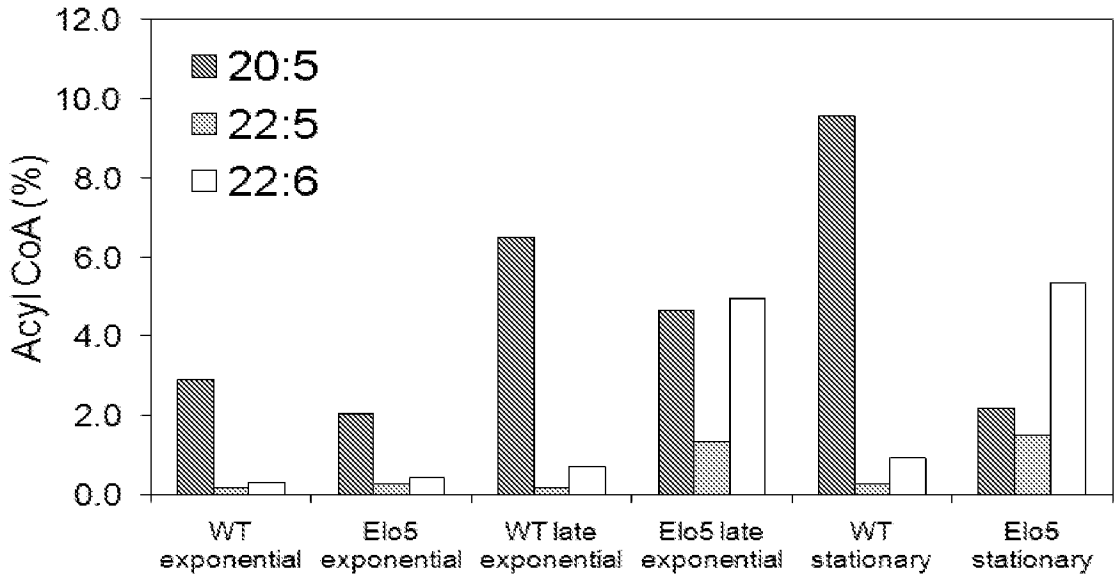


FIGURE 5a

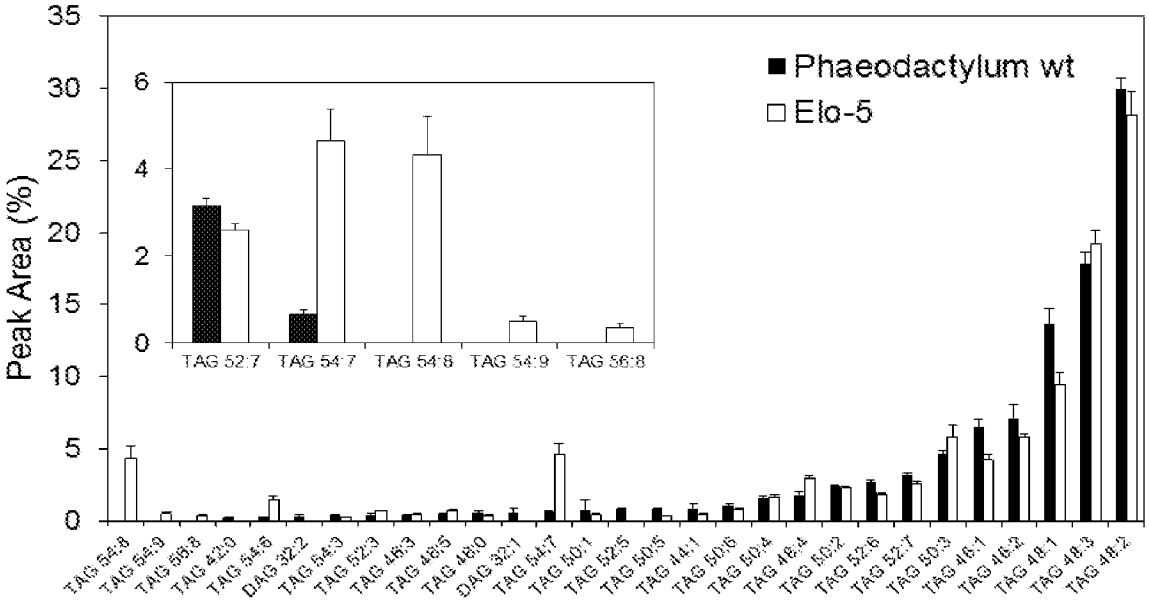


FIGURE 5b

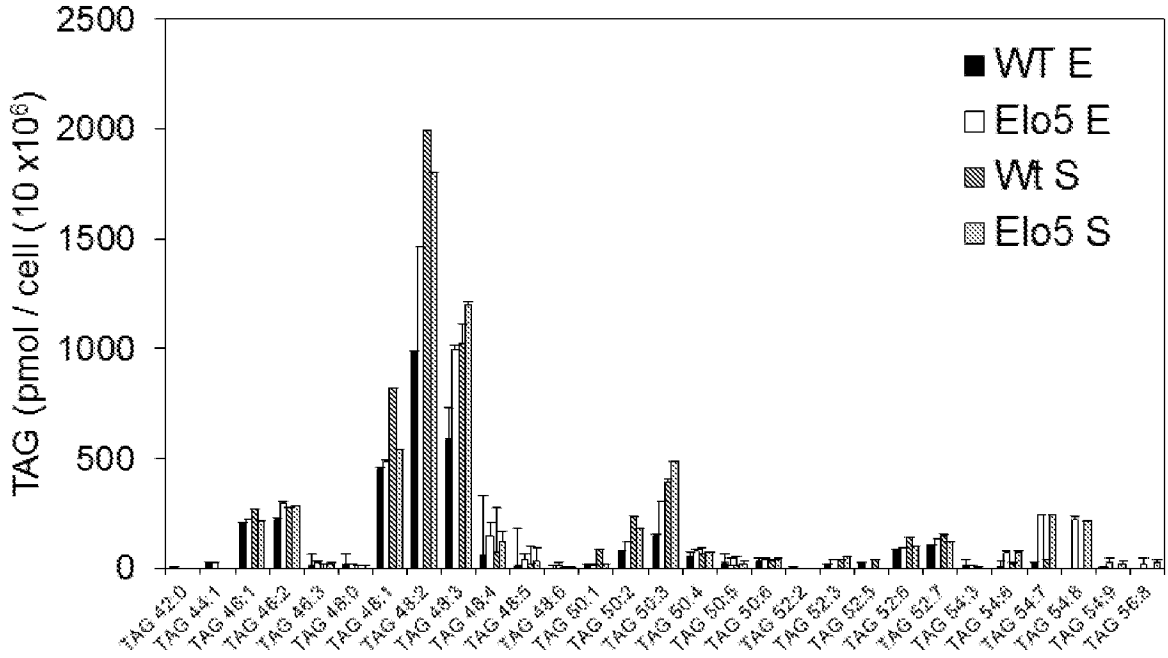


FIGURE 6a

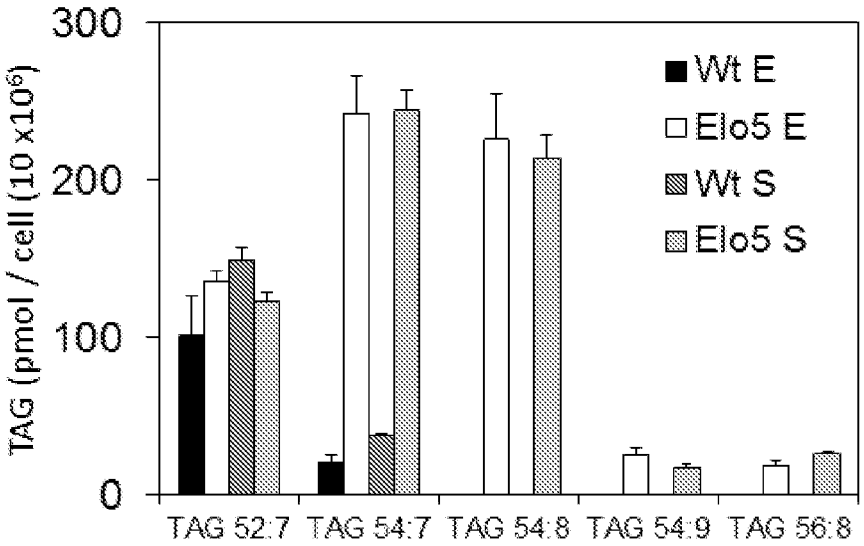


FIGURE 6b

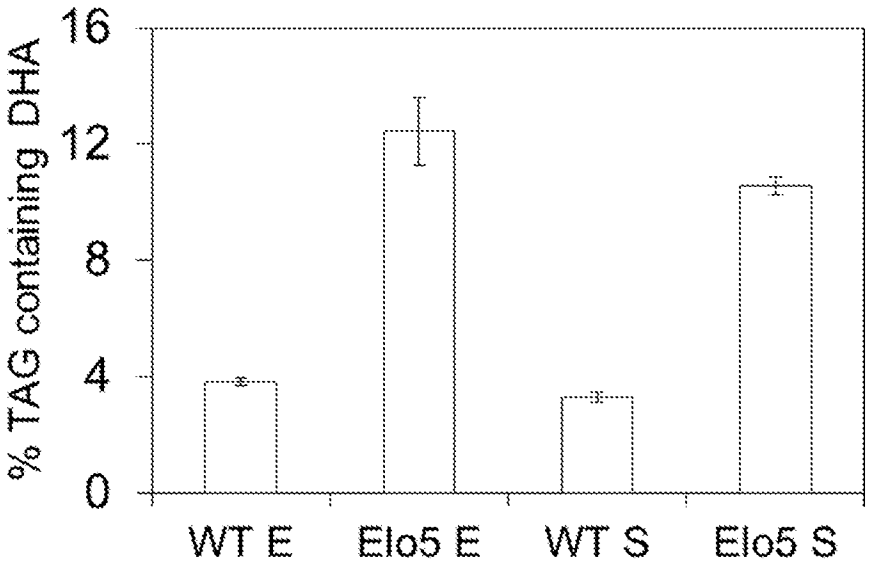
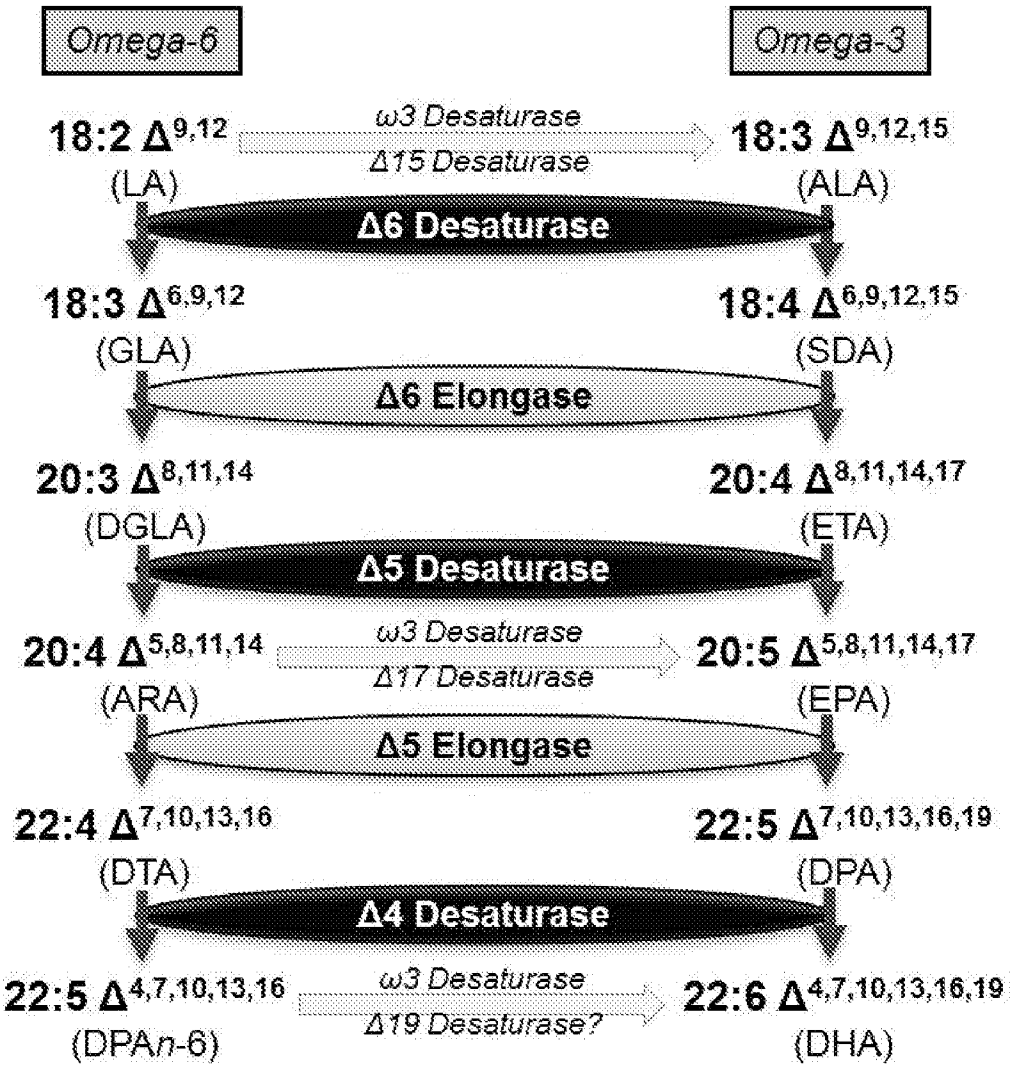


FIGURE 7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Omega-3
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(LA)
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Δ15 Desaturase
18:3 Δ9,12,15
(ALA)
Δ6 Desaturase
18:3 Δ6,9,12
(GLA)
18:4 Δ6,9,12,15
(SDA)
Δ6 Elongase
20:3 Δ8,11,14
(DGLA)
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(ETA)
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20:4 Δ5,8,11,14
(ARA)
ω3 Desaturase
Δ17 Desaturase
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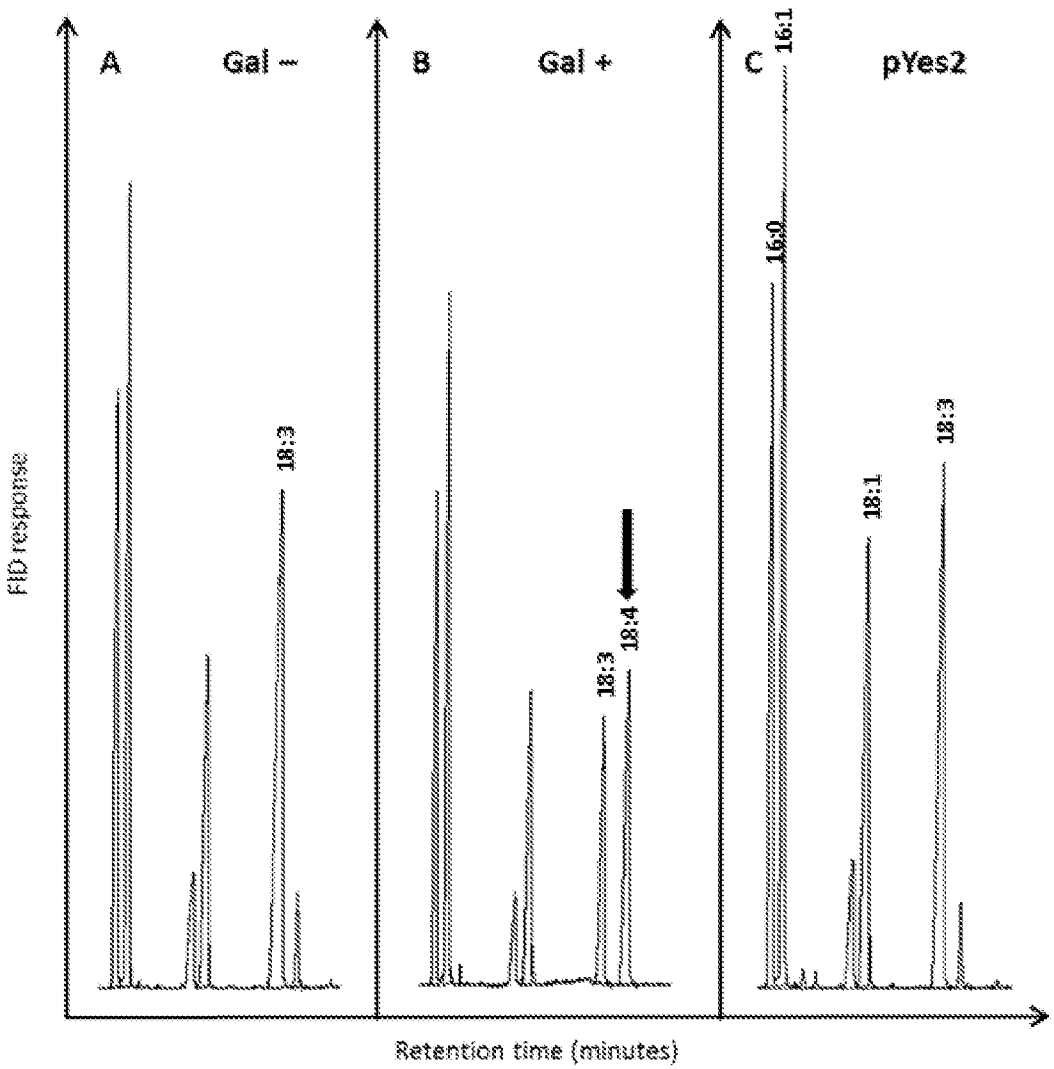
(DHA)

FIGURE 8


A
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B
Gal +
C
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16:0
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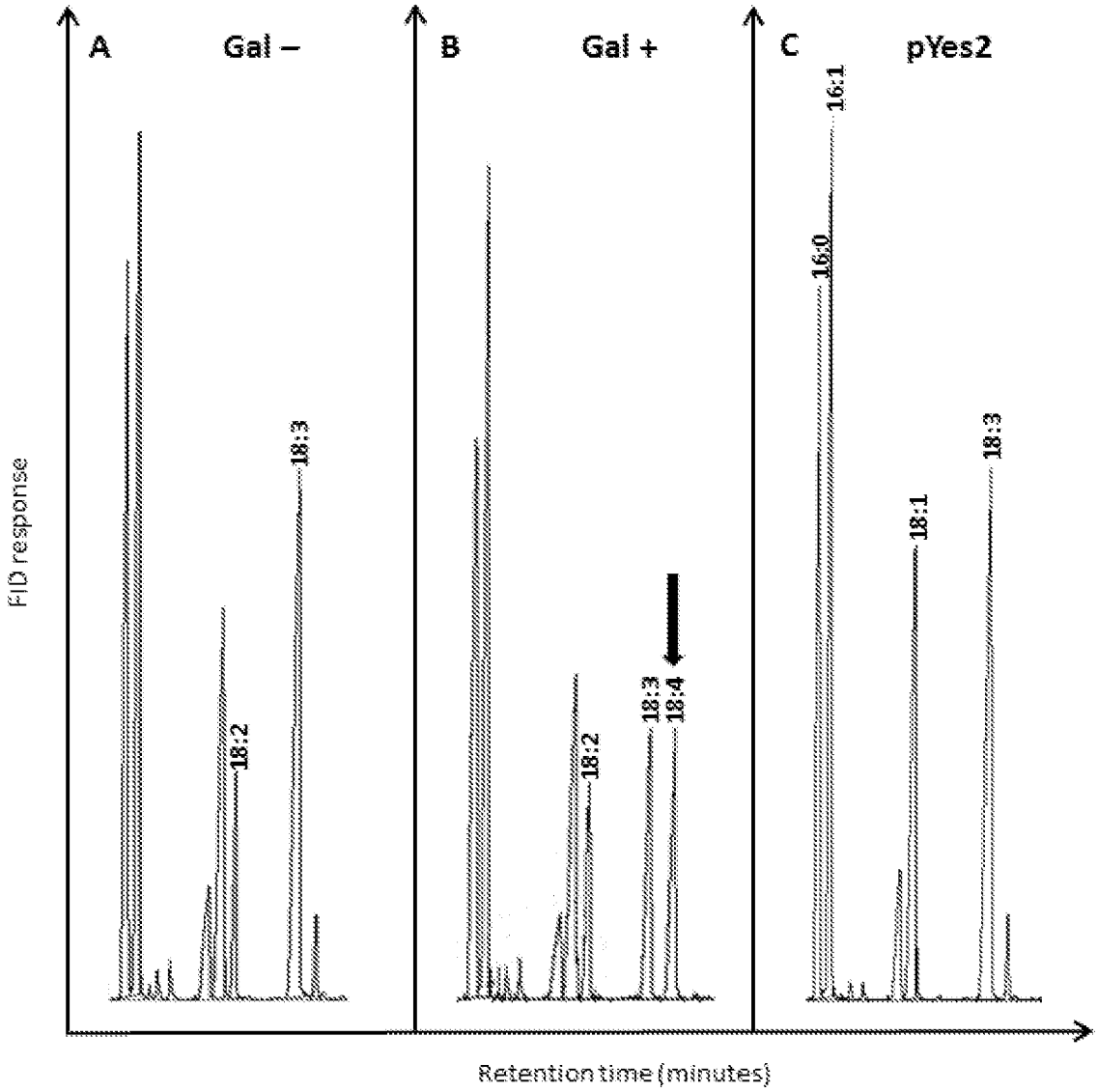
18:1
18:3
18:4
18:3
FID response
Retention time (minutes)

FIGURE 9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Gal +
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18:2
16:1
16:0
18:1
18:3
Retention time (minutes)

FIGURE 10

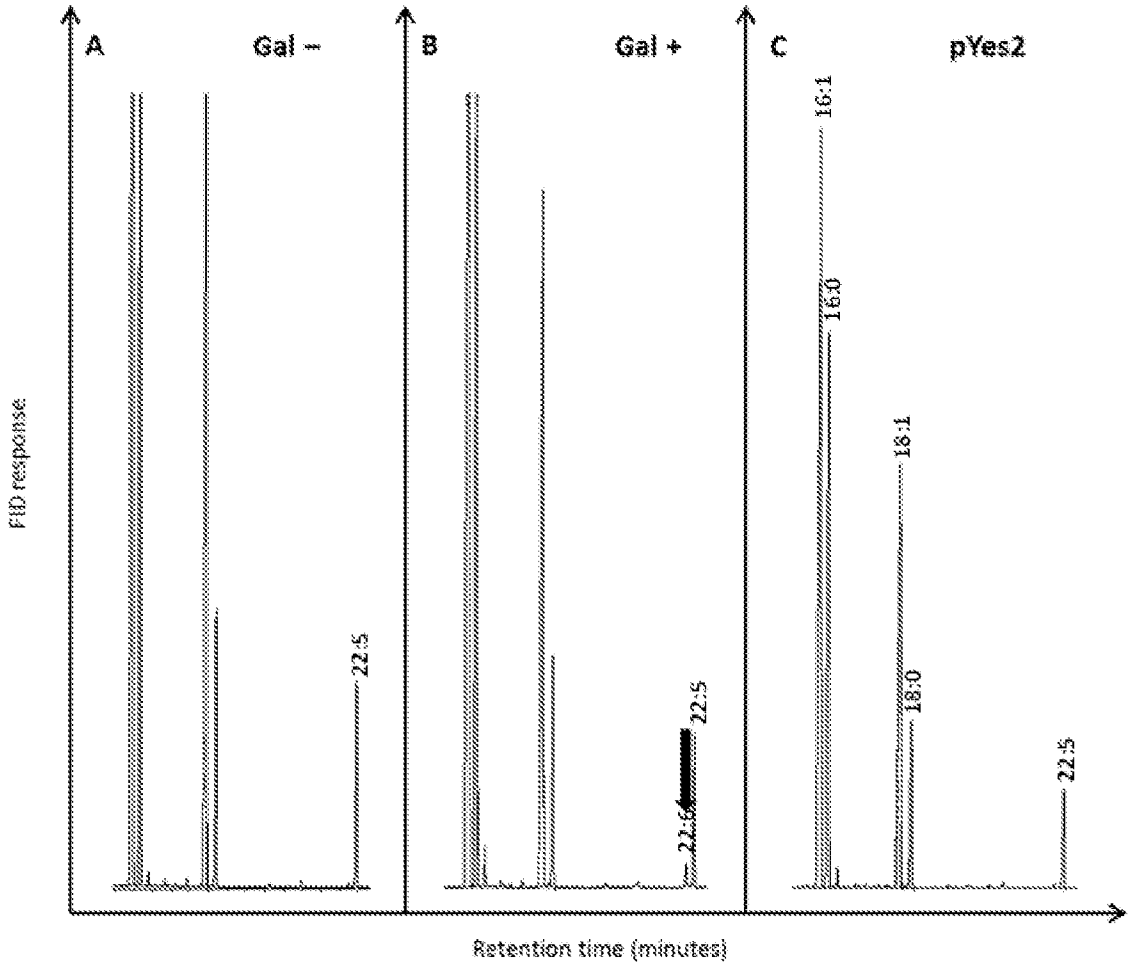

A
Gal -
B
Gal +
C
pYes2
FID response
16:1
16:0
18:1
18:0
22:5
22:5
22:6
22:5
Retention time (minutes)

FIGURE 11

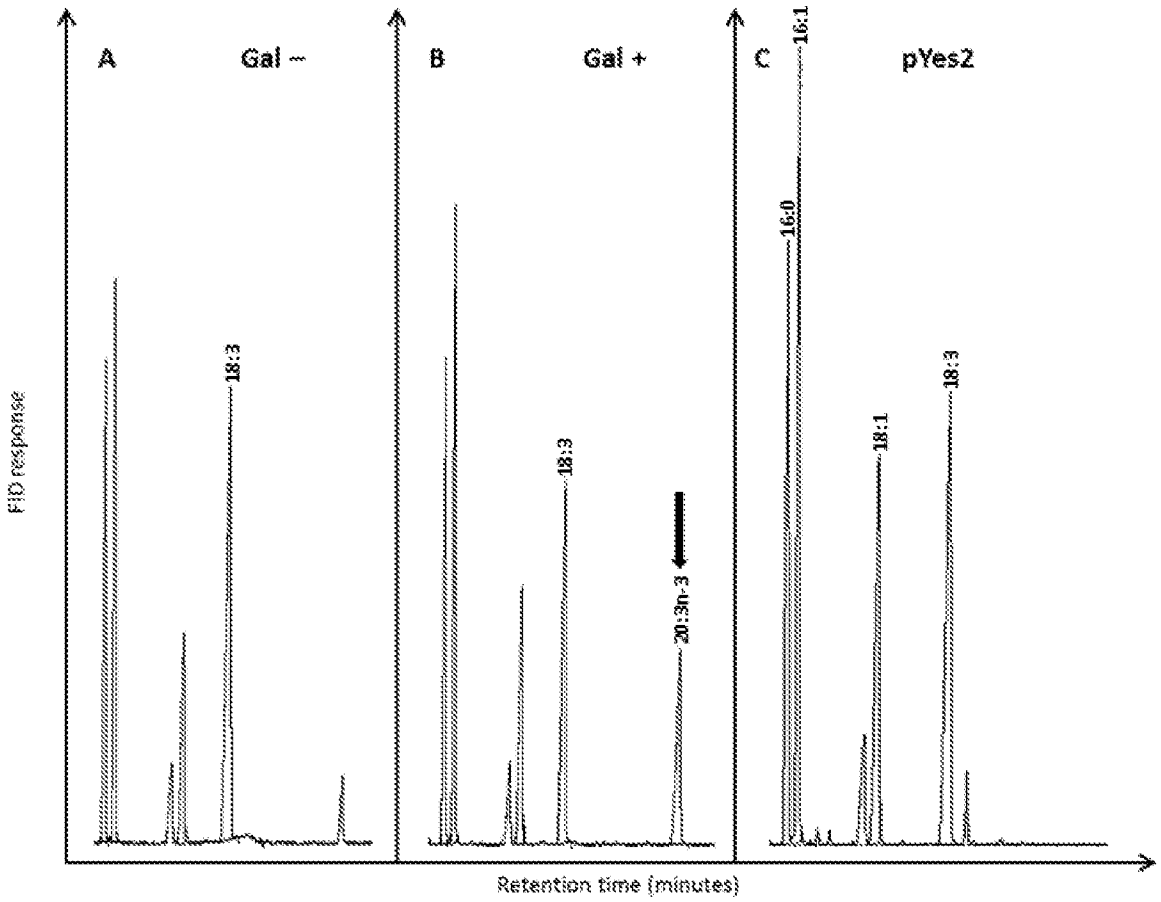

A
Gal –
B
Gal +
C
pYes2
16:1
16:0
18:3
18:3
18:1
18:3
20:3n-3
FID response
Retention time (minutes)

FIGURE 12

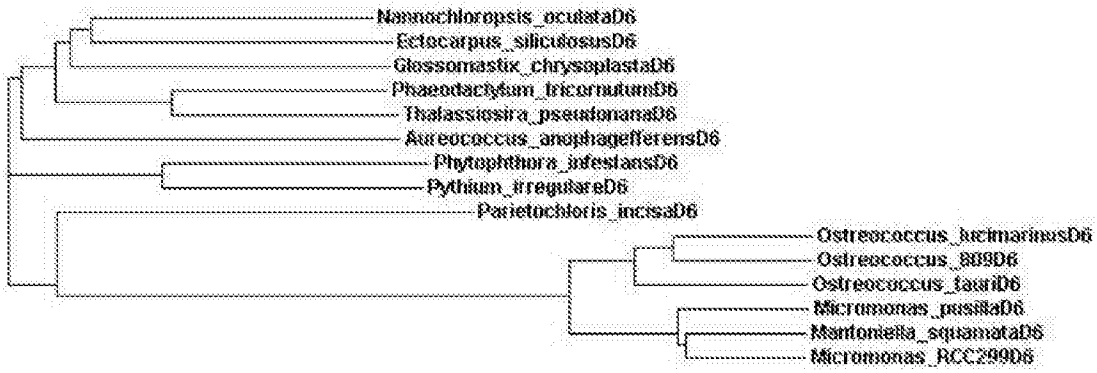


FIGURE 13

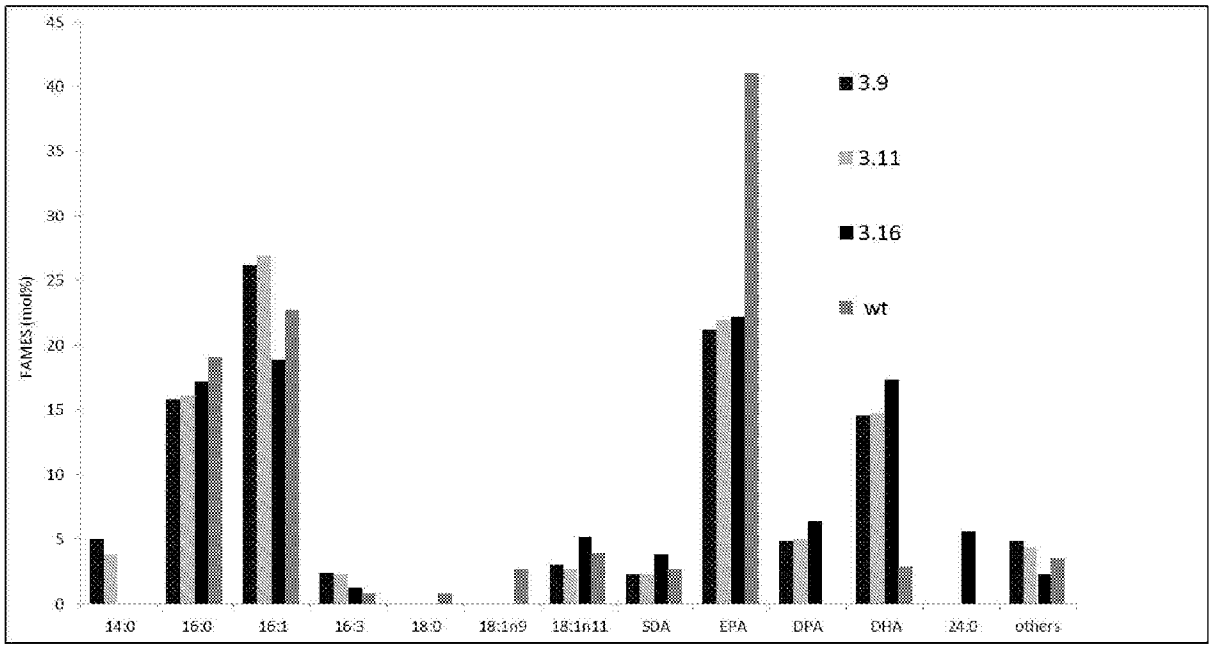


FIGURE 14

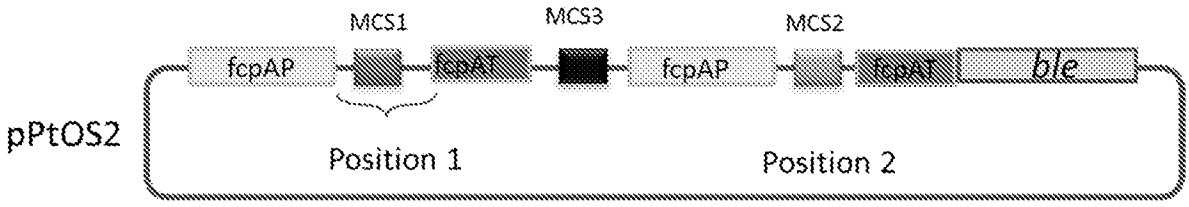


FIGURE 15

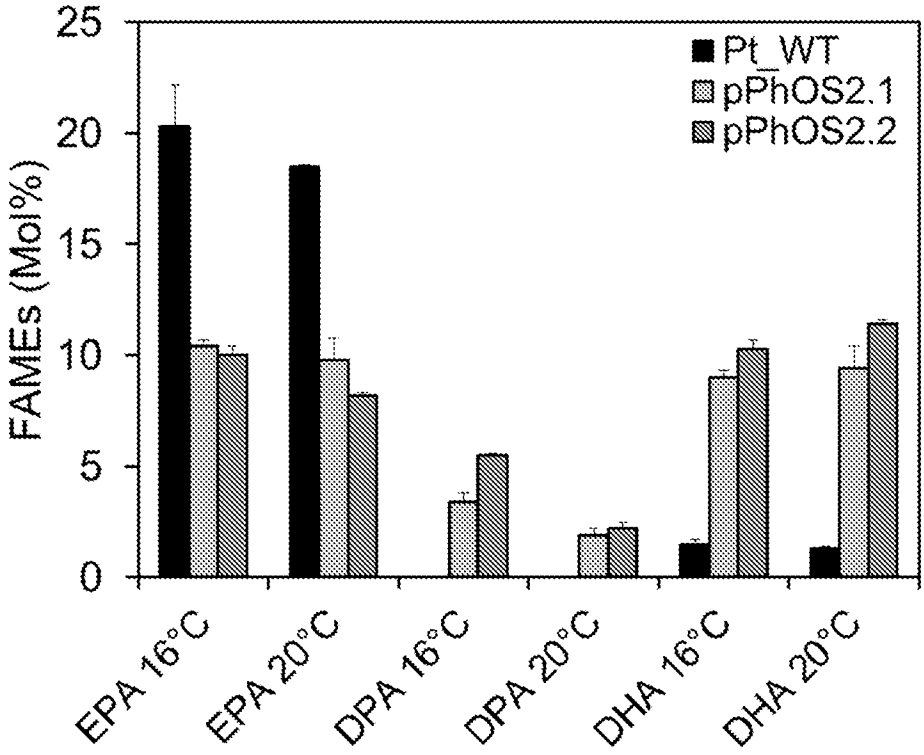


FIGURE 16

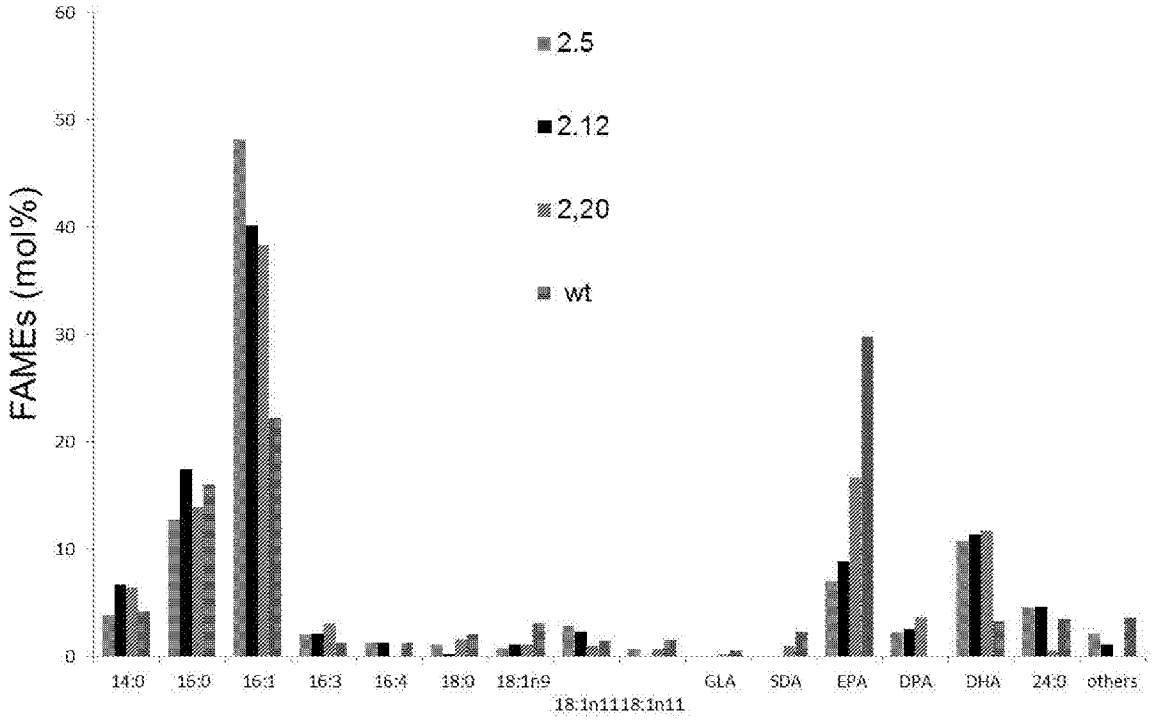

FAMEs (mol%)
60
50
40
30
20
10
0
2.5
2.12
2,20
wt
14:0
16:0
16:1
16:3
16:4
18:0
18:1n9
18:1n11
18:1n11
GLA
SDA
EPA
DPA
DHA
24:0
others

FIGURE 17

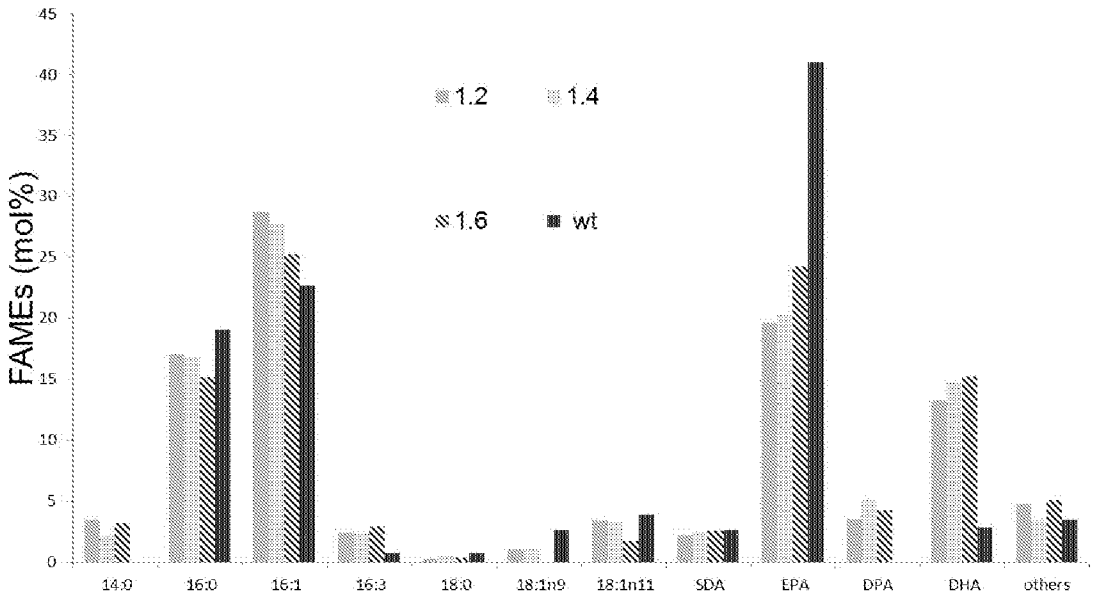
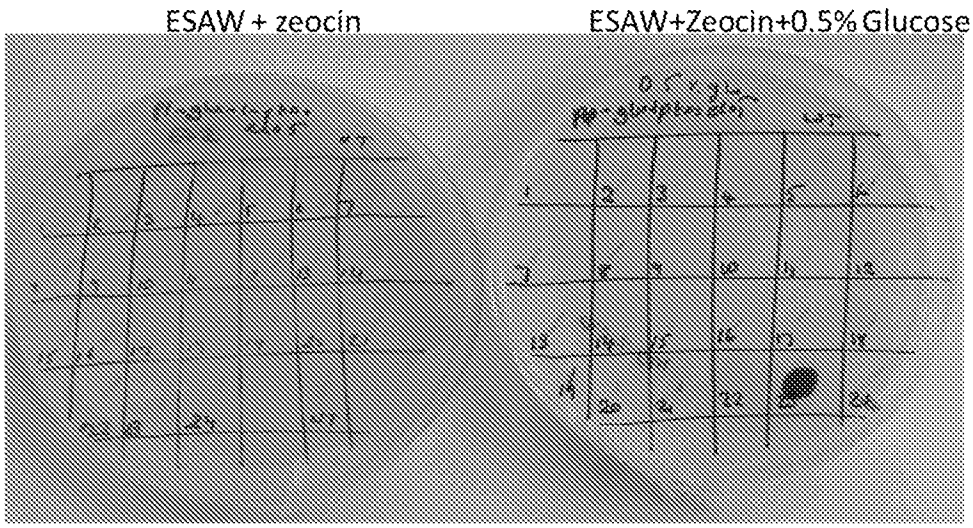


FIGURE 18



Dark grown plates +/- glucose 10 days after single colonies were streaked on to plates
WT cells cannot grow in the dark (top of plates)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052553

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/057246 A1 (COMMW SCIENT IND RES ORGANISAT [AU]; PETRIE JAMES ROBERTSON [AU]; MACK) 27 May 2010 (2010-05-27) page 1 - page 170; claims 1-175	1-44
X	WO 2011/161678 A2 (UNIV BEN GURION [IL]; HACOHEN ZVI [IL]; KHOZIN GOLDBERG INNA [IL]; UMI) 29 December 2011 (2011-12-29) page 1 - page 53; claims 1-21	1-44

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2013	28/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brochado Garganta, M

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUANG Y-S ET AL: "Enzymes for transgenic biosynthesis of long-chain polyunsaturated fatty acids", BIOCHIMIE, MASSON, PARIS, FR, vol. 86, no. 11, 1 November 2004 (2004-11-01), pages 793-798, XP004689088, ISSN: 0300-9084, DOI: 10.1016/J.BIOCHI.2004.09.019 page 793 - page 796	1-44
X	MEYER A ET AL: "NOVEL FATTY ACID ELONGASES AND THEIR USE FOR THE RECONSTITUTION OF DOCOSAHEXAENOIC ACID BIOSYNTHESIS", JOURNAL OF LIPID RESEARCH, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC, US, vol. 45, no. 10, 1 October 2004 (2004-10-01), pages 1899-1909, XP009046591, ISSN: 0022-2275, DOI: 10.1194/JLR.M400181-JLR200 page 1899 - page 1908	1-44
X	ZHOU ET AL: "Isolation and characterization of genes from the marine microalga Pavlova salina encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis", PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 68, no. 6, 3 March 2007 (2007-03-03), pages 785-796, XP005912642, ISSN: 0031-9422, DOI: 10.1016/J.PHYTOCHEM.2006.12.016 page 785 - page 795	1-44
A	WARD O P ET AL: "Omega-3/6 fatty acids: Alternative sources of production", PROCESS BIOCHEMISTRY, ELSEVIER, NL, vol. 40, no. 12, 1 December 2005 (2005-12-01), pages 3627-3652, XP027794053, ISSN: 1359-5113 [retrieved on 2005-12-01] the whole document	1-44

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052553

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARWOOD J L ET AL: "The versatility of algae and their lipid metabolism", BIOCHIMIE, MASSON, PARIS, FR, vol. 91, no. 6, 1 June 2009 (2009-06-01), pages 679-684, XP026119774, ISSN: 0300-9084, DOI: 10.1016/J.BIOCHI.2008.11.004 [retrieved on 2008-11-27] the whole document -----	1-44
A	RUBIO-RODRIGUEZ N ET AL: "Production of omega-3 polyunsaturated fatty acid concentrates: A review", INNOVATIVE FOOD SCIENCE AND EMERGING TECHNOLOGIES, ELSEVIER, AMSTERDAM, NL, vol. 11, no. 1, 1 January 2010 (2010-01-01), pages 1-12, XP026825026, ISSN: 1466-8564 [retrieved on 2010-01-05] the whole document -----	1-44
A	WO 2011/054800 A1 (DSM IP ASSETS BV [NL]; VERKOEIJEN DANIEL [US]; BIJL HENDRIK LOUIS [NL]) 12 May 2011 (2011-05-12) the whole document -----	1-44

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2013/052553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010057246 A1	27-05-2010	AR 074364 A1	12-01-2011
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		CA 2743880 A1	27-05-2010
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		WO 2011054800 A1	12-05-2011