# Enhancing the Production of Omega-3 Polyunsaturated Fatty Acids in Marine Diatoms

Royah Vaezi

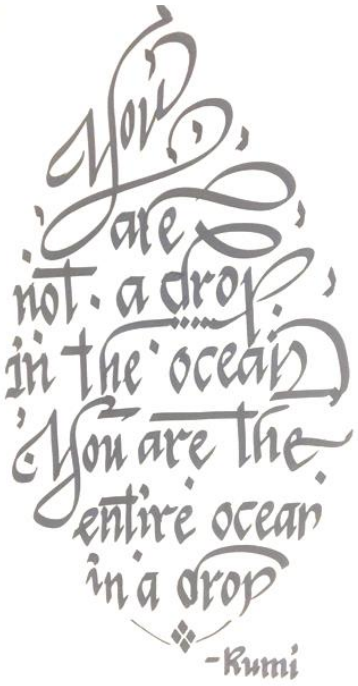
B.Sc., ARCS, M.Res., DIC

A thesis submitted for the degree of Doctor of Philosophy

University of East Anglia, Norwich, UK

School of Environmental Sciences

January 2015


You
are
not a drop
in the ocean
You are the
entire ocean
in a drop
-Rumi

*For my family*

# Abstract

The primary producers of essential omega-3 polyunsaturated fatty acids are marine microalgae, which form the base of the aquatic food web. One alternative source of our ever-diminishing stocks of fish and fish oil is via the cultivation of these microorganisms. Unfortunately, these microalgae, of which diatoms are the dominant class, only accumulate oil during specific stages of their life-cycle and/or under nutritional states which are incompatible with the required high density of growth and target fatty acid profile. Additionally, the endogenous levels of desirable fatty acids such as eicospantaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3) are usually relatively modest (in the range of 10-35% of total fatty acids) and therefore present an opportunity for enhancement.

A database search carried out on the genomes of omega-3-producing unicellular photoautotrophic green alga *Ostreococcus* sp. RCC809 and cold-water diatom *Fragilariopsis cylindrus* led to the identification of four novel genes involved in omega-3 biosynthesis. These genes encoded an omega-3-specific Δ6-desaturase, a Δ4-desaturase, a Δ6-elongase and a Δ5-elongase.

Overexpression of genes encoding Δ6-desaturase and Δ6-elongase activities in *Thalassiosira pseudonana* impacted the fatty acid and acyl-CoA profiles of this model centric diatom. Changes to chloroplast and lipid droplet phenotype were also observed.

Targeted knock-down of native genes involved in the omega-3 biosynthetic pathway was carried out in *T. pseudonana* to further understand endogenous omega-3 fatty acid production. Cells targeted for the knock-down of Δ9-desaturase exhibited a drastically altered growth phenotype, but maintained a wild type-like fatty acid profile. This phenotype was attributed to the possibility of another, functionally redundant, protein that escaped sequence-based silencing, masking the knock-down of Δ9-desaturase.

The results and observations provided in this thesis contribute new valuable information to the field of lipidomic research in microalgae, breaking new ground in metabolic engineering of lipid metabolism in diatoms.

# Table of Contents

# List of Tables

# List of Figures

# Preface

Essential omega-3 polyunsaturated fatty acids are synthesised primarily in the marine environment, and accumulate in the aquatic food web through consumption (Williams, 2006). Marine microbes, particularly diatoms, are the primary producers of these essential fatty acids, which are vital for human health but cannot be synthesised *de novo* (Nakamura et al, 2004). Omega-3 fatty acids have numerous benefits in treating human diseases such as cardiovascular disease (CVD) (Masson et al, 2007), neurological disorders (Freeman et al, 2006), obesity (Buckley & Howe, 2009) and cancers (Siddiqui et al, 2008). Furthermore, the omega-3 fatty acid docosahexaenoic acid (DHA) has been shown to be a crucial factor in brain and eye development (Innis, 2008). Although vegetable oils are also nutritionally valuable, they do not deliver the same health benefits as fish oils, thus making fish oils a valuable commodity with many different applications, including use as dietary supplements.

The current major source of omega-3 fatty acids is fish oils. As supplements these have undesirable flavours and odours, as well as having varying unguaranteed quality. The high demand for omega-3 fatty acids has also put a strain on the fishing industry (Pauly et al, 2005). Large-scale culturing of aquatic microbes that produce omega-3 fatty acids is undertaken for high value applications such as for use in infant formula milks, but are difficult to scale-up and are a particularly costly (Lee, 2001; Agostoni, 2008).

There is a growing need for alternative sustainable sources of the essential omega-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The advancement of our understanding of microalgal lipid metabolism, including the characterisation of genes involved in omega-3 biosynthetic pathways and the factors regulating them, will enable the development of industrially compatible large-scale sustainable systems for the production of omega-3 fatty acids. The results and observations provided in this thesis contribute new valuable information to this field of research.

The main objectives of this doctoral research project are threefold:

1) **To identify and functionally characterise algal genes involved in omega-3 biosynthesis.** This serves multiple purposes: firstly to provide genes that can be heterologously expressed in other organisms/other algal species; secondly, to expand the microalgal knowledge bank by providing gene/protein/behavioural information on a variety of functions from different sources; and finally, to further the understanding of the processes that govern omega-3 biosynthesis in microalgal species.

2) **To metabolically engineer *T. pseudonana* to enhance omega-3 production and/or accumulation.** In the first instance this will be attempted by the overexpression of heterologous genes. This study will also help to elucidate further information about lipid metabolic processes in *T. pseudonana*, with the aim of contributing further insight into lipid metabolism in microalgae.

3) **To silence/impair endogenous genes involved in omega-3 biosynthesis in *T. pseudonana*.** This study will be carried out with the aim of determining whether silencing of targeted genes has a positive or negative effect on omega-3 production/accumulation. Also to further understand the native omega-3 biosynthetic processes in *T. pseudonana*, again providing valuable insights into lipid metabolism in this diatom.

# Acknowledgments

This work would not have been possible without the efforts of a number of people, for whom I am extremely grateful.

Firstly, thank you to Professor Thomas Mock , Professor Johnathan Napier and Dr Olga Sayanova for giving me the opportunity to undertake such a fascinating and stimulating PhD project, and the BBSRC and IBTI Club for providing the funding for this to happen.

I feel very fortunate to have been able to learn from and receive advice from Johnathan, Olga and Thomas. Specifically, it has been invaluable working as part of Johnathan's team at Rothamsted and I am grateful to Olga for her day-to-day guidance and support. Also, thank you to Johnathan for supporting me with all the additional projects I undertook alongside my PhD, such as BiotechnologyYES at Syngenta and EnterpriseWISE and the University of Cambridge. Thank you to Thomas for introducing me to the intriguing world of diatoms and for sharing his extensive knowledge in this area. Also for his continued advice and support over the past four years.

I would like to thank Rachel Hipkin, Jan Strauss and Amy Kirkham at UEA for all their help in the lab on my visits up to Norwich and for always answering emails with any algal-related experimental queries I had. Special thanks also to Jan for cell pellets of *Fragillariopsis cylindrus* used in Chapter 2.

Thank you to my colleagues in Lab 115 at Rothamsted Research. In particular, it was a pleasure to work so closely with Mary Hamilton and I really appreciated all the advice and our many chats.

Also at Rothamsted, my mentor Smita Kurup and Kirstie Halsey in Bioimaging for all her help with the confocal microscopy. Special thanks to Ian and Helen for keeping the lab running with all of their technical help.

Astrid, Omar and Luis – we went through the PhD experience together and it definitely wouldn't have been the same without the three of you.

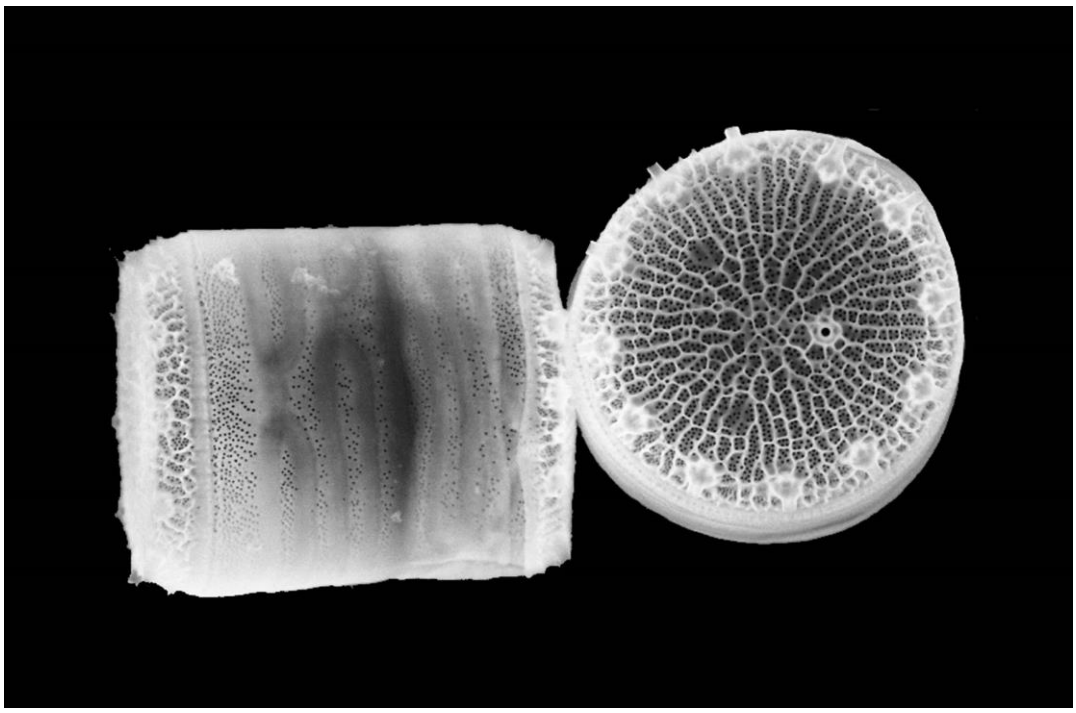
Friends of The Manor – I have so many wonderful memories with all of you and I'm already planning my round-the-world trip to visit those of you who are scattered across the globe now!

I would like to thank my friends in "the world outside of PhD" for putting up with my obsession with algae and my anti-socialness in the last few months so that I could tend to my experiments and thesis.

To Paolo – words are not enough, but I will try. The past four years have been a momentous journey for the both of us, which we shall surely never forget. Thank you for being with me every step of the way throughout my PhD, for offering your advice, for reading every single word of this thesis, for being a formatting genius… Put simply, thank you for everything.

To my family – thank you from the bottom of my heart for always being there for me, providing endless love, support and encouragement. It means a lot to me that you've all read (or at least attempted to!) my paper and I'm sure you'll give it a go with this thesis as well. I hope I've made you proud.

And finally – *Thalassiosira pseudonana*. After seemingly endless flasks of cultures, we got there in the end. In a strange way, I may even miss them.



**SEM image of *T. pseudonana* taken by Professor Nils Kröger and published in the article 'The Genome of the Diatom Thalassiosira pseudonana: Ecology, Evolution and Metabolism' in the journal 'Science', October 2004.**

# Chapter 1

# Introduction

## 1.1 LIPIDS: SYNTHESIS AND ACCUMULATION IN MICROALGAE

Many microalgae are able to synthesise and accumulate lipids, particularly in the form of neutral storage lipids such as triacylglycerol (TAG). Large amounts of TAG can be accumulated under environmental stress conditions. TAGs are a valuable lipid class with potential application for fuels, chemicals, and nutraceuticals. Important fatty acids, such as the essential omega-3 polyunsaturated fatty acids (PUFAs), can be incorporated into TAG. In order to engineer microalgae to enhance omega-3 PUFA production and accumulation it is important to understand the biosynthetic pathways behind these processes.

### 1.1.1 Introduction to Fatty Acids and Lipids

Fatty acids are carboxylic acids with a long unbranched aliphatic chain which can be saturated or unsaturated. They have the general chemical formula R – COOH (where R is the hydrocarbon chain and COOH is the carboxylic acid group). The majority of naturally occurring fatty acids have an even chain length. In plants and animals the fatty acid hydrocarbon chain generally consists of 12 – 22 carbon atoms. Fish and marine mammals typically have fatty acids predominantly with chain lengths of 20 and 22 carbon atoms, whereas terrestrial animals and plants are more abundant in fatty acids with chain lengths of 18 carbons and lower.

Three sub groups make up the fatty acid family – saturated fatty acids, monoenoic or monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). The distinguishing factor in these groups is the presence or absence, and number, of double bonds in the aliphatic chain. Saturated fatty acids contain no double bond between the carbon atoms and are typically solid or semi-solid at room temperature. Monounsaturated fatty acids contain only a single double bond in the carbon chain and are usually liquid at room temperature, but can become semi-solid at low temperatures. Polyunsaturated fatty acids include two or more double bonds in the aliphatic chain. PUFAs which have a hydrocarbon chain of 20 or more carbon atoms are commonly known as LC-PUFAs (long-chain polyunsaturated fatty acids). In algae, the *de novo* synthesis of fatty acids occurs in the

chloroplast. In animals and fungi, however, this process takes place in the cytosol (Ohlrogge and Browse, 1995).

Fatty acids can be considered as the components of lipids. When they are not attached to lipids they are known as free fatty acids. Free fatty acids yield large quantities of ATP when metabolised so can be considered as an important source of energy. Most of the fatty acids that are synthesised in plants and algae are esterified with glycerol.

Lipids can contain more than one fatty acid in their composition. Lipids with attached fatty acids are saponifiable. Such lipids include waxes, phospholipids, glycolipids and triacyclglycerols. Phospholipids form lipid bilayers and so are key components of all cell membranes. Waxes consist of long alkyl chains and can include esters of carboxylic acids and long chain alcohols, or mixtures of primary alcohols and fatty acids. They are insoluble in water. Glycolipids consist of a lipid with an attached carbohydrate moiety. They are important in cell recognition as well as in the provision of energy. Triacyglycerols (TAGs) are lipids formed from glycerol esterified with long chain fatty acids on all three positions of the glycerol backbone. The glycerol backbone of membrane glycerolipids, however, is typically esterified with fatty acids at the sn-1 and sn-2 positions, with a polar or non-polar head group at the sn-3 position.

TAG serves as an energy store and this energy can be released rapidly on demand. TAGs also function as a reserve of essential fatty acids and eicosanoid precursors. Within all cell types TAGs are stored as cytosolic lipid droplets. Algal oil production is much greater than that of terrestrial crops, with some algal species accumulating up to 60% TAG (dry cell weight) (Hu et al, 2008). In algae it is generally believed that TAG is synthesised via a direct glycerol pathway, known as the Kennedy pathway. The Kennedy pathway involves fatty acid production in the chloroplast, followed by the sequential transfer from Coenzyme-A (CoA) to the sn-1, sn-2 and sn-3 positions on the glycerol-phosphate (Figure 1).

Lipid biosynthetic pathways have been well characterised in higher plants, mammals and fungi (Ohlrogge and Browse, 1995; Pereira et al, 2003; Bergen and Mersmann, 2005; Guschina and Harwood, 2006), however, the specific mechanisms involved in algal lipid biosynthesis are yet to be fully elucidated. Work has been done to uncover genes and enzymes involved in various pathways, as well as their characterisation, but significant research is still required in order to fully understand lipid biosynthesis in microalgae. Lipid biosynthesis in algae is most likely to be similar to what is known to occur in higher plants,

therefore pathways in the latter offer a valuable guide in order to clarify similar mechanisms in microalgae.

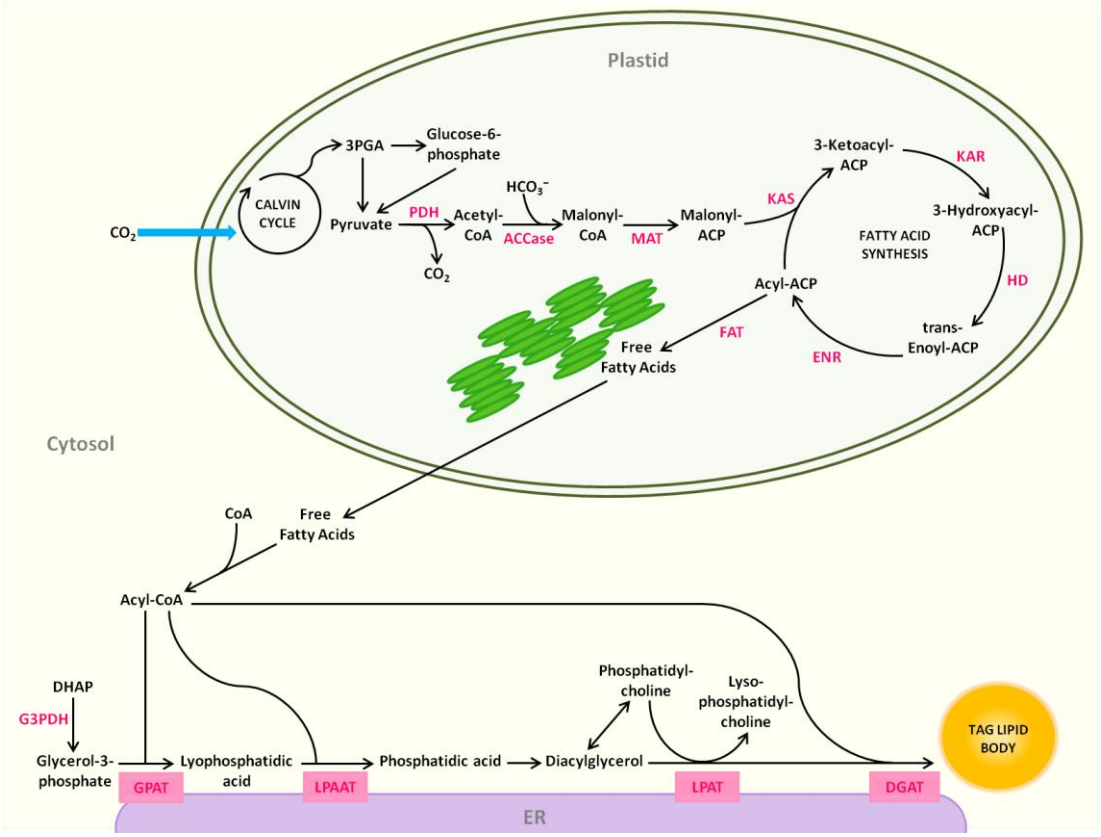



**Figure 1:** General overview of lipid metabolism in microalgae. The plastid is the site of free fatty acid biosynthesis. TAG lipid bodies may be assembled at the ER. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, gycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerol.

### 1.1.2 Introduction to Omega-3 Polyunsaturated Fatty Acids

Omega-3 fatty acids are a class of LC-PUFAs. LC-PUFAs consist of a chain of 20 or more carbon atoms and three or more methylene- interrupted double bonds at the cis position of the fatty acid chain. There are two classes of LC-PUFA – omega-6 (n-6) and omega-3 (n-3). The position of the double bond proximal to the methyl end of the carbon chain determines which family the fatty acid belongs to (Figure 2). In the case of omega-3 fatty acids, the first double bond

in the chain is between the third and fourth carbon atoms from the methyl group. In the omega-6 family, on the other hand, the first double bond is between the sixth and seventh carbon atoms in the chain. Each class of fatty acid is synthesised in a stepwise pathway consisting of alternating elongation and desaturation reactions (Figure 3). In the omega-6 biosynthetic pathway linoleic acid (18:2n-6, LA) undergoes sequential Δ6-desaturation, Δ6-elongation and Δ5-desaturation resulting in the production of arachidonic acid (20:4n-6, ARA). In the omega-3 pathway alpha-linolenic acid (18:3n-3, ALA) follows the same desaturation and elongation reactions leading to the formation of eicosapentaenoic acid (20:5n-3, EPA). In the traditional pathway omega-6 and omega-3 pathways can be interconnected by a ω3 desaturase reaction converting ARA into EPA, so omega-3 fatty acids can be synthesised from the omega-6 precursors. Further Δ5 elongation and Δ4 desaturation reactions of EPA leads to the synthesis of docosapentaenoic acid (22:5n-3, DPA) and docosahexaenoic acid (22:6n-3, DHA).

Omega-3 and omega-6 fatty acids are metabolically distinct and have opposing physiological functions in humans. Omega-3 fatty acids play a vital role in human health. Humans, like other animals, are unable to synthesise the omega-3 fatty acids *de novo* (Nakamura and Nara, 2008). Omega-3 fatty acids are therefore determined as “essential” fatty acids since they must be obtained through dietary intake. They are vital functional components of the cell membrane regulating structure, fluidity and permeability; but not just in a structural capacity, they also have importance in human metabolism as precursors of bioactive molecules such as eicosanoids, metabolites which help to regulate inflammation, plaque aggregation, immune-reactivity and vasoconstriction and dilation. ARA and DHA have been shown as crucial in neonatal health and development, in particular with regards to brain development and the acquirement of ocular vision. As a result ARA and DHA have been recommended to be included in infant formula milks. EPA and DHA have been recognised through clinical studies as having protective effects against metabolic syndrome, including the related obesity and type-2 diabetes disease states, cancers, atherosclerosis, cardiovascular disease, dementia, childhood attention-deficit hyperactivity disorder and mental illnesses, particularly depression. Omega-3 fatty acids have also been shown to confer beneficial effects to the human immune system, reproductive system, in the resolution of inflammation and in skin barrier function.

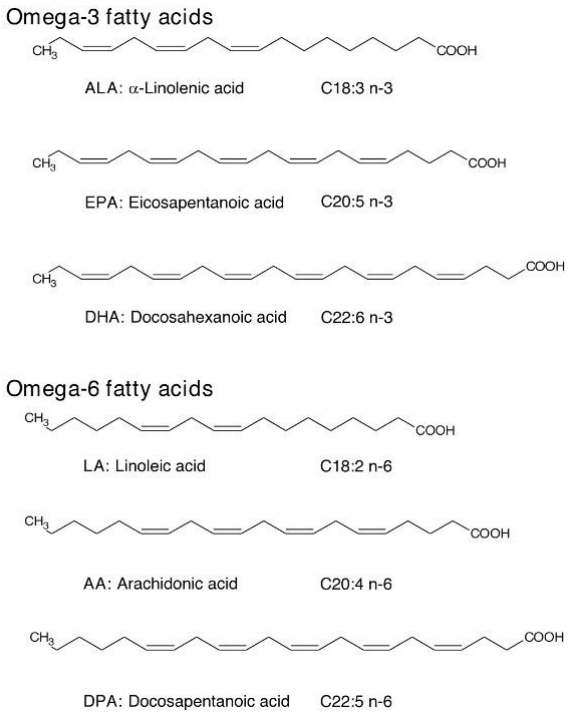



**Figure 2:** Comparison of a selection of omega-3 and omega-6 polyunsaturated fatty acid chains.

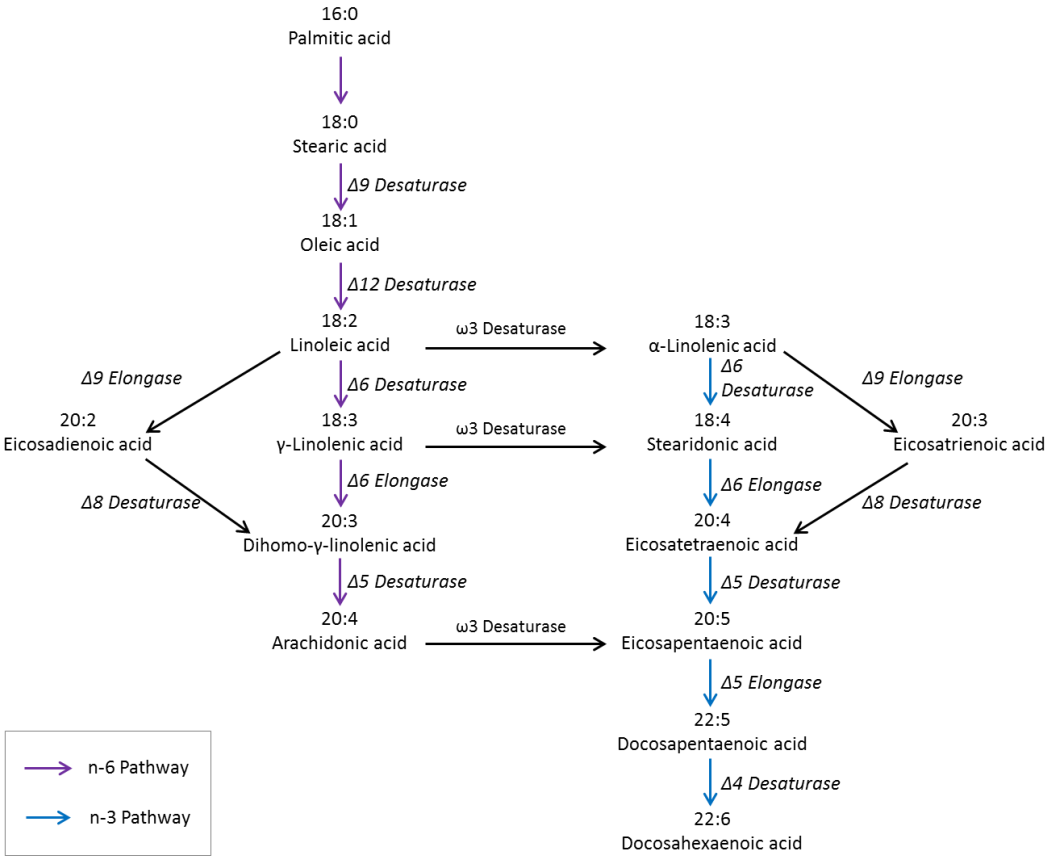



**Figure 3:** Conventional fatty acid biosynthesis pathways. The n-6 pathway is shown on the left, and the n-3 pathway on the right.

### 1.1.3 Fatty Acid Biosynthesis

#### 1.1.3.1 Acetyl-CoA carboxylase

The first committed step in fatty acid synthesis is the ATP-dependent formation of malonyl-CoA from the irreversible carboxylation of acetyl-CoA and bicarbonate, catalysed by acetyl-CoA carboxylase (ACCase). ACCase is a multi-subunit enzyme found in most prokaryotes and in the chloroplasts of plants and algae. In eukaryotes it is a large multi-domain enzyme located in the endoplasmic reticulum. ACCase is not found in the Archaea kingdom.

Two types of ACCases have been identified in plants; the first a heteromeric enzyme located in the chloroplast and the second a multifunctional homomeric enzyme located in the cytosol (Cronan Jr. and Waldrop, 2002). The first type of ACCase is suggested to catalyse most of the production of malonyl-CoA for fatty acid synthesis, whereas the second type is suggested to catalyse the synthesis of cuticular lipid and flavonoid biosynthesis precursors in the cytosol of higher plants (Ohlrogge and Browse, 1995; Harwood, 1996).

In *E. coli*, the genes encoding ACCase subunits have been cloned and characterised; these subunits include the α-carboxyltransferase (accA), biotin carboxyl carrier protein (accB), biotin carboxylase (accC) and β-carboxyltransferase (accD). Homologous ACCase subunits have been identified in plants and algae, with the observation that accA, accB and accC subunits are nuclear-encoded and the accD subunit is chloroplast/plastid-encoded (Cronan Jr. and Waldrop, 2002). ACCases from algae and most higher plants are similar, they are composed of the same multiple subunits, each of which is a single multifunctional peptide containing biotin carboxylase, biotin binding, carboxyltransferase, and ATP-binding domains (Roessler, 1990). The rate-limiting ACCase-catalysed carboxylation of acetyl-CoA to malonyl-CoA occurs in two steps: i) the biotin carboxylase subunit of ACCase transfers $CO_2$ to the nitrogen atom of the biotin prosthetic group, ii) carboxyltransferase catalyses the transfer of the activated $CO_2$ from biotin to acetyl-CoA, forming malonyl-CoA (Ohlrogge and Browse, 1995).

ACCases have been purified from the diatom *Cyclotella cryptica* (Roessler, 1990) and the marine haptophyte *Isochrysis galbana* (Livne & Sukenik, 1990). The *I. galbana* ACCase had a molecular mass of approx. 700 kDa, similar to higher plant ACCases. The *C. cryptica* ACCase was 740 kDa. The gene encoding the *C. cryptica* ACCase has been cloned and characterised (Roessler and Ohlrogge, 1993), revealing interesting observations. The accB domain was found to be less conserved than the accA, accC and accD domains, with the latter exhibiting

strong similarity to yeast and animal ACCase subunits. The N-terminus of the *C. cryptica* ACCase amino acid sequence has a signal peptide which imports the ACCase into the chloroplast via the endoplasmic reticulum (ER). A number of experiments in various plant and algal species revealed that ACCase is a rate-limiting factor of fatty acid biosynthesis (Harwood, 1996; Ohlrogge and Jaworski, 1997). ACCase undergoes complex transcriptional and post-translational regulation due to its essential role in many cellular metabolic processes (Ohlrogge and Jaworski, 1997; Madoka et al, 2002; Sasaki and Nagano, 2004). Its activity is regulated by the redox state of ferredoxin through disulphate-dithiol exchange between the α and β subunits of carboxyltransferase, pH and magnesium ions (Kozaki et al, 2001). Overexpression of ACCase in *C. cryptica* and *Navicula sapuvila* (Dunahay et al, 1996) didn't result in increased lipid production, despite increased ACCase activity (Sheehan et al 1998). One explanation that has been suggested for this is from observations in the oleaginous microalga *Nannochloropsis oceanica*, is that the transcriptional control of ACCase to the partitioning of fatty acids into TAG is less significant than genes involved in the Kennedy pathway (Hu et al, 2014; Wang et al, 2014).

#### 1.1.3.2 Fatty acid synthase

The malonyl-CoA produced from the carboxylation of acetyl-CoA by ACCase is the central carbon donor for fatty acid synthesis. Catalysed by fatty acid synthase (FAS) (Harwood, 1996), the malonyl group is transferred to the co-factor protein acyl carrier protein (ACP) as a thioester, which then undergoes a series of condensation reactions with acyl-ACP or acetyl-CoA acceptors to extend the acyl chain (Ohlrogge and Browse, 1995), also catalysed by FAS, leading to the synthesis of palmitate. ACP is required to covalently bind all fatty acyl intermediates.

There are two classes of FAS. Type I FAS systems are common in mammals and fungi, comprising a single large multifunctional polypeptide, whereas dissociated type II FAS systems are common in archaea and bacteria and consist of discrete single-function proteins translated from separate genes (Chan and Vogel, 2010). Despite this variation, the individual enzyme reactions that occur in the two FAS systems are identical (Smith and Sherman, 2008).

Plant and algal FAS belong to the type II system. The initial FAS reaction transferring malonyl to ACP is catalysed by the condensing enzyme 3-ketoacyl ACP synthase III (KAS III) (Jaworski et al, 1989). Most of the subsequent condensation reactions are catalysed by KAS I and KAS II catalyses the conversion of palmitate to stearate (Harwood, 1988). Successive fatty acid chain elongation occurs with three additional reactions: a 3-ketoacyl ACP reductase (KAR)

catalysed reductive step, a hydroxyacyl ACP dehydratase (HD) catalysed dehydration step and a final enoyl ACP reductase (ENR) catalysed reductive step. Each FAS cycle of reactions leads to the extension of the fatty acid chain by two carbon atoms, usually ending with the synthesis of saturated C16:0- and C18:0-ACP (Ohlrogge and Browse, 1995). KAS enzymes may begin to limit FAS rates under high flux through ACCase (Postbeittenmiller et al, 1992). Genome and transcriptome sequencing of a number of oleaginous *Nannochloropsis* strains suggests that FAS may be involved in transcriptional control of lipid synthesis (Hu et al, 2014; Wang et al, 2014; Li et al, 2014). However, experiments overexpressing one of the KAS isoforms resulted in little or no effect on fatty acid synthesis, likely due to other KAS isoforms or cofactors, for example malonyl-CoA, becoming rate limiting (Dehesh et al, 2001). The observation that saturated acyl-ACP intermediates are found in similar quantities in plant leaves and seeds suggests that all of the KAS enzyme activities are likely to be identical (Postbeittenmiller et al, 1992).

#### 1.1.3.3 Chain length and saturation

Fatty acid chain elongation is terminated either by the removal of the acyl group from ACP by a chloroplast acyltransferase, transferring newly synthesised fatty acids from ACP to glycerol-3-phosphate (G3P), or by the hydrolysis of acyl-ACP by an acyl-ACP thioesterase, releasing free fatty acids (Ohlrogge and Browse, 1995). The first reaction, catalysed by chloroplast acyltransferases, known as the prokaryotic pathway, produces glycerolipids with a C16 fatty acid at the sn-2 position of the glycerol backbone (Browse and Somerville, 1991). A glycerol-3- phosphate acyltransferase (GPAT) in the chloroplast competes with acyl-ACP thioesterase for the acyl-ACP. The second reaction, catalysed by acyl-ACP thioesterase, results in the hydrolysis of acyl-ACPs producing free fatty acids in the inner envelope of the chloroplast. These free fatty acids are exported to the external side of the outer envelope and are re-incorporated by long-chain fatty acyl-CoA synthetases, forming acyl-CoAs for use in cytosolic glycerolipid synthesis (Chapman and Ohlrogge, 2012). The precise mechanism for this export step is unknown, however, fatty acid transport could occur via protein-mediated transport of nascent fatty acids released on the chloroplast inner envelope to long-chain fatty acyl-CoA synthetases on the outside of the chloroplast (Koo et al, 2004). An alternative explanation is that acyl groups may be incorporated into phosphatidylcholine (PC) in the chloroplast envelope before being transported to the ER via a direct membrane contact site. It would appear that PC has a central role in lipid synthesis due to the fact that the majority of newly formed fatty acids are incorporated into PC before other glycerolipids (Bates et al, 2007), and also the rapid exchange of acyl groups between PC and acetyl-CoA pools providing

precursors for *de novo* glycerolipid synthesis (Chapman and Ohlrogge, 2012). However, since some algal lipidomes lack PC, for example some strains of *C. reinhardtii* (Sakurai et al, 2014), the exact mechanism of fatty acid export in algae is still ambiguous and requires further investigation.

There are two classes of acyl-ACP thioesterases: FatA which hydrolyses unsaturated acyl-ACPs, and FatB which hydrolyses saturated acyl-ACPs (Jones et al, 1995). In a recent study on *Nannochloropsis oceanica* IMET1, under nitrogen deprivation transcriptional expression of FatA increased 2.7-fold at 24 hours and 3.1-fold at 48 hours (Li et al, 2014), suggesting that in this organism, FatA preferentially releases C16:0 from acyl-ACP, and therefore contributes to the accumulation of TAG containing C16:0 fatty acids.

In the so-called eukaryotic pathway (Harwood, 1996), cytosolic acyltransferases produce glycerolipids from the acyl-CoA pool of fatty acids. The re-esterification of free fatty acids to acyl-CoA occurs through long-chain fatty acyl-CoA synthetases. Substrate specificity of cytosolic acyltransferases results in the formation of eukaryotic glycerolipids, with C18:0 at the sn-2 position of the glycerol backbone. The transfer of lipids from the eukaryotic pathway in the ER to chloroplast envelopes, known as retrograde transfer, has been extensively studied in plants, but has not been observed in the model alga *C. reinhardtii* (Benning, 2008; Benning, 2009).

Very long chain fatty acids (20 or more carbons) can be produced from the elongation of existing long-chain acyl-CoAs, by fatty acid elongase complexes using cytosolic malonyl-CoA (Harwood, 1996). The saturated fatty acids produced *de novo* can undergo desaturation reactions either by plastidic desaturases or ER desaturases. In vascular plants, the plastidic stearoyl-ACP desaturase (Δ9-desaturase) introduces a double bond at the Δ9 of stearoyl-ACP, forming 18:1-ACP. Δ9-desaturase is a soluble enzyme located in the stroma and important in determining the ratio of saturated to unsaturated fatty acids. This ratio must be tightly regulated to maintain membrane fluidity and is therefore vital under extreme light and temperature conditions (Harwood, 1996). In the green alga *Haematococcus pluvialis*, it was observed that when Δ9-desaturase and the biotin carboxylase subunit of ACCase genes are upregulated in response to high light conditions, they are expressed at constant molar ratios during the accumulation of fatty acids and the keto-carotenoid astaxanthin (Chen, 2007). This would indicate that there is coordinated regulation of fatty acid synthesis and desaturation by ACCase and Δ9-desaturase.

In plants, studies of *Arabidopsis* mutants led to the identification of a number of loci responsible for lipid desaturation. 18:1 and 18:2 desaturases undertaking lipid desaturation in the ER are encoded by the *fad2* and *fad3* genes, respectively (FAD – fatty acid desaturase). Desaturases responsible for lipid desaturation in the chloroplast are encoded by *fad4*, *fad5*, *fad6*, *fad7* (Ohlrogge and Browse, 1995). Recent studies have indicated the presence of *fad*-like genes in microalgae (An et al, 2013).

#### 1.1.3.4 Galactoglycerolipid degradation in algal plastids

Experiments in *C. reinhardtii* have revealed that some *de novo* synthesised fatty acids are incorporated into monogalactosyldiacylglycerol (MGDG), a plastid lipid, before transportation to the cytosol for TAG synthesis (Li et al, 2012). This MGDG turnover pathway is separate from those described above which are catalysed by acyltransferases or thioesterases. Li and colleagues observed that disruption of the plastid galactoglycerolipid degradation 1 (PGD1) gene resulted in a TAG-less phenotype. *In vivo* pulse-chase labelling experiments revealed that galactolipid, particularly MGDG, is a major source of fatty acids incorporated into TAG under low nitrogen conditions. Furthermore, in the PGD1 mutant, there was reduced fatty acid flux from plastid fatty acids to TAG. The authors used these results to propose a model for PGD-dependent TAG synthesis in microalgae, whereby PGD plays a central role in MGDG turnover and provides plastidic fatty acid precursors for TAG synthesis in the ER. The model PGD catalysed pathway doesn't account for all these precursors in microalgae, however, which suggests a combination of pathways involving MGDG turnover, and the thioesterase- and acyltransferase-dependent pathways.

### 1.1.4 TAG Biosynthesis

#### 1.1.4.1 The Kennedy pathway

In eukaryotes TAG synthesis occurs via the Kennedy pathway, through a series of three sequential acylation reactions of G3P. In microalgae, phosphatidic acid (PA) and diacylglycerol (DAG) intermediates from this pathway are used for the synthesis of membrane polar lipids, which affect reproduction and growth. Few enzymes involved in the Kennedy pathway have been characterised in microalgae. However, the study of this pathway in microalgae is greatly aided by its characterisation in other organisms, particularly higher plants. The outline of the Kennedy pathway below is based on this knowledge with

the assumption that it will provide a framework for lipid metabolism and TAG accumulation in microalgae.

#### 1.1.4.1.1 Glycerol-3-phosphate acyltransferase

The first committed step in the Kennedy pathway is the transfer of a fatty acid from the acyl-CoA pool, or acyl-ACP, to the sn-1 or sn-2 position of G3P. This reaction, catalysed by glycerol-3-phosphate acyltransferase (GPAT), results in the production of lysophosphatidic acid. In plants and algae there are two types of GPAT – chloroplast GPAT and extraplastidic GPAT.

In mammals a four-member gene family encodes GPATs – *gpat1*, *gpat2*, *gpat3*, *gpat4*. Cytological studies in a number of organisms revealed that GPAT1 and GPAT2 are localised to the outer mitochondrial membranes, and the GPAT3 and GPAT4 isoforms are localised to the ER (Hammond et al, 2002; Kalinowski et al, 2003; Zhuan et al., 2006; Nagle et al, 2008; Chen et al, 2008; Wendel et al, 2009). GPAT1 and GPAT3 are believed to play essential roles in the synthesis of TAG (Hammond et al, 2002; Kalinowski et al, 2003; Zhuan et al, 2006), as is GPAT4, based on loss-of-function experiments (Nagle et al, 2008). Studies on the higher plant *Arabidopsis thaliana* have revealed nine GPAT encoding genes within the genome. GPATs 1 – 8 from *A. thaliana* are from a plant-specific evolutionary lineage and several of these GPAT enzymes are involved in polymer synthesis (e.g. cutin and suberin) (Zheng et al, 2003; Shockey et al, 2006; Beisson et al, 2007; Li et al, 2007). The ER-associated GPAT9 enzyme from *A. thaliana* is highly conserved with the mammalian GPAT3. Observations of the upregulation of GPAT9 during embryogenesis in *A. thaliana* suggest its involvement in TAG biosynthesis (Shockey et al, 2006).

Compared with higher plants and mammals, microalgae generally contain fewer copies of extraplastidic GPAT-encoding genes, as indicated by genome-wide surveys of these organisms (Li et al, 2013). Algal GPATs may be responsible for the synthesis of extraplastidic membrane glycerolipids as well as TAG. Li and colleagues report experiments in *C. reinhardtii* where a single gene encoding an extraplastidic GPAT has been cloned and partially characterised. Under stress conditions, e.g. high light or nitrogen deprivation, upregulation of the *gpat* gene accompanies TAG accumulation. Knock-down of this gene in *C. reinhardtii* resulted in a reduction of TAG, but its heterologous overexpression in *Saccharomyces cerevisiae* led to increased TAG production. These observations confirm that in this organism GPAT is responsible for TAG and extraplastidic membrane lipid synthesis, which may well be the case in other microalgae (Li et al, 2013). Another membrane-bound GPAT from the

diatom *Thalassiosira pseudonana* was found to have a high preference for 16:0-CoA substrates (Jia et al, 2009).

The chloroplast GPAT is a soluble protein and has only been fully characterised in *A. thaliana*. It acylates G3P to lysophosphatidic acid and could be responsible for galactolipid synthesis (Kunst et al, 1998). Much of the TAG found in a number of eukaryotic microalgae contains a 16:0 at the sn-2 position on the glycerol backbone (Sukenik et al, 1993; Fan et al, 2011), suggesting that full characterisation of algal GPATs could provide further understanding of TAG biosynthesis through the prokaryote pathway.

#### 1.1.4.1.2 Lysophosphatidic acid acyltransferase

Lysophosphatidic acid acyltransferase (LPAAT) catalyses the esterification of lysophosphatidic acid at the sn-2 position of the glycerol backbone, giving rise to phosphatidic acid (PA). PA is a precursor for TAG and membrane phospholipid biosynthesis (Coleman and Lee, 2004).

There are five genes predicted to encode LPAATs in *A. thaliana*, with the confirmed functionality of LPAAT1, LPAAT2 and LPAAT3 (Kim et al, 2005). LPAAT1 encodes a plastidic isoform and is essential for embryo development in *A. thaliana*, as determined by *in vitro* experiments. LPAAT1 has high substrate specificity for 16:0-CoA, which is consistent with the higher level of plastidic glycerolipids with this moiety at the sn-2 position, compared with 18:1-CoA (Wagner et al, 2004). LPAAT2, however, prefers 18:1-CoA to 16:0-CoA substrates, which is a characteristic trait of an enzyme involved in the eukaryotic pathway. Studies on LPAAT2 found it to be localised to the ER and revealed that it is essential for female, but not male, gametophyte development in *A. thaliana* (Kim et al, 2005). Kim and colleagues also found that LPAAT3, present in pollen, is similar to LPAAT2 and displays a preference for 18:1-CoA.

Experiments overexpressing a microsomal LPAAT from rapeseed in *A. thaliana* resulted in enhanced TAG content in seeds (Maisonneuve et al, 2010). It has been previously established that some seed-specific LPAAT proteins are able to catalyse the acylation of the sn-2 position of seed storage TAG with uncommon acyl groups, such as C12 in coconut (Oo and Huang, 1989) and C22 in meadowfoam (Laurent and Huang, 1992). These observations suggest LPAAT may be a promising target for the manipulation of TAG acyl composition in higher plants.

Soluble cytosolic LPAAT in *A. thaliana* can also catalyse the acylation of lysophosphatidic acid (Ghosh et al, 2009). It is a multifunctional enzyme exhibiting TAG lipase and PC hydrolysis activities. Disruption of the gene encoding this LPAAT in *A. thaliana* resulted in a tenfold increase of TAG in loss-of-function mutants, compared to control plants, as well as the accumulation of lipid droplets in mature leaves (James et al, 2010). This study suggests that this soluble cytosolic LPAAT is a lipase rather than acyltransferase, however, targeted manipulation of this enzyme's activities could potentially be used to enhance TAG in higher plants or algae.

An extraplastidic LPAAT has only been identified in a *Micromonas* species, and not in the model alga *C. reinhardtii* or other microalgae. It has been suggested that a GPAT with broad substrate specificity, or a divergent protein in algae, may catalyse this reaction (Merchant et al., 2012). In *C. reinhardtii*, homologues of plant ER-type acyltransferases (GPAT and LPAAT) and phosphatidic acid phosphatase are found to be single enzymes, predicted to possess N-terminal chloroplast-targeting signals (Tardif et al. 2012).

#### 1.1.4.1.3 Phosphatidic acid phosphatase

Diacylglycerol (DAG) is a precursor for TAG, phospholipid (Coleman and Lee, 2004) and galactolipid synthesis (Benning and Ohta, 2005). It is produced from the dephosphorylation of PA by phosphatidic acid phosphatase (PAP). At least two types of PAP exist in *A. thaliana*, a prokaryotic and eukaryotic PAP. Three isoforms of prokaryotic PAP, thought to be localised to the chloroplast, have been identified and are homologous to an ancestral PAP in the primitive anaerobic phototrophic bacterium *Chlorobium tepidum* (Nakamura et al, 2007). Two eukaryotic PAP isoforms have been identified; PAP-1, $Mg^{2+}$-dependent and thought to be involved in glycerolipid synthesis, and PAP-2, believed to play a role in signal transduction (Coleman and Lee, 2004). In *A. thaliana* two genes encoding PAP-1, *pah1* and *pah2*, were found to be highly expressed during late seed development, when there is a high demand for glycerolipids. Disruption of these genes resulted in a decrease in the synthesis of galactolipids (Nakamura et al, 2009; Eastmond et al, 2010). It is unclear whether *pah1* and *pah2* are essential for TAG synthesis in *A. thaliana*, however, their homologues in animals and yeast have been demonstrated to play an important role in such a process (Peterfry et al, 2001; Carmen and Henry, 2007; Han et al, 2006).

In *C. reinhardtii*, two candidate PAP-encoding genes have been identified, both of which showed transcriptional upregulation in response to nitrogen deprivation (Miller et al, 2010). Due to the suggested pivotal role of the prokaryotic pathway in the *de novo* synthesis of TAG,

the plastidic PAP may be particularly important for TAG biosynthesis in *C. reinhardtii* (Merchant et al, 2012).

#### 1.1.4.1.4 Diacylglycerol acyltransferase

The final step of the Kennedy pathway for the production of TAG is the acylation of 1-2-diacylglycerol with an acyl-CoA, catalysed by diacylglycerol acyltransferase (DGAT) (Ohlrogge and Browse, 1995). DGAT may be crucial in controlling fatty acid flux to storage TAG. Several types of DGAT have been identified, including a bifunctional DGAT/wax ester synthase from *Acinetobacter calcoaceticus* (Kalscheuer and Steinbuchel, 2003), type 1 DGAT (DGAT1) and type 2 DGAT (DGAT2) (Lung and Weselake, 2006), a soluble cytosolic DGAT from peanut cotyledons (Saha et al, 2006) and a *Euonymus* DGAT which catalyses the synthesis of 3-acetyl-1,2-diacyl-sn-glycerols (Durrett et al, 2010). DGAT1 and DGAT2 are responsible for most of the TAG biosynthesis in the majority of organisms (Durrett et al, 2010).

In *A. thaliana* DGAT1 contributes significantly to TAG synthesis in seeds. Its overexpression leads to enhanced TAG accumulation (Zou et al, 1997; Jako et al, 2001). DGAT1 is a multifunctional enzyme belonging to the membrane-bound O-acyltransferase family, which exhibit broad substrate specificities. *In vitro* studies of mouse DGAT1 revealed a broad range of acyltransferase activities, including the production of DAG (acyl-CoA:monoacylglycerol acyltransferase), production of wax esters (wax monoester and wax diester synthase activities) and production of retinyl esters (acyl-CoA:retinol acyltransferase) (Nguyen et al, 2005).

DGAT2 is generally smaller than DGAT1, with two transmembrane domains. It was first discovered in the oleaginous fungus *Mortierella ramanniana* (Lardizabal et al, 2001), followed by the identification of DGAT2 encoding genes in higher plants. Studies of DGAT2 in plants have shown that it is strongly induced in developing seeds at the initiation of oil biosynthesis (Shockey et al, 2006).

DGAT2 is ubiquitous in eukaryotic algae, as determined from the comparative analysis of available algal genomes (Wagner et al, 2010), with most known species containing multiple isoforms. Four isoforms of DGAT2 have been identified in *T. pseudonana*, five in *P. tricornutum*, six in *C. reinhardtii* and eleven in *N. oceanica*. In *C. renhardtii* two of the isoforms are upregulated during nitrogen deprivation (Miller et al, 2010; Boyle et al, 2012). Two isoforms of DGAT2 in the unicellular green alga *Ostreococcus tauri* can lead to the formation of TAG, as confirmed by yeast-based functional assays (Wagner et al, 2010). DGAT2 isoforms

from few microalgae have been functionally characterised: *P. tricornutum* (Gong et al. 2013; Guiheneuf et al. 2011), *O. tauri* (Wagner et al. 2010), *C. reinhardtii* (Boyle et al. 2012; Miller et al. 2010; Msanne et al. 2012; Hung et al. 2013) and recently in *Neochloris oleoabundans* (Chungjatupornchai and Watcharawipas, 2014). Gain-of-function and loss-of-function studies in *C. reinhardtii* have revealed the involvement of one of the isoforms in TAG biosynthesis (Boyle et al, 2012; Deng et al, 2012; La Russa et al, 2012). Recently, expression of a DGAT2 from *Brassica napus* in *C. reinhardtii* resulted in enhanced lipid production (Ahmad et al, 2014).

#### 1.1.4.2 Galactolipid:galactolipid galactosyltransferase

An alternative pathway for the biosynthesis of TAG in plants is the transglycosylation of MGDG to oligogalactolipids and DAG by galactolipid:galactolipid galactosyltransferase (GGGT). In *A. thaliana* the *sfr2* gene has been found to encode GGGT (Moellering et al, 2010). SFR2 is essential for freezing tolerance. In *sfr2* mutants undergoing freeze treatment there is an approximate 50% reduction in TAG, compared to wild type (Moellering et al, 2010). Furthermore, newly synthesised TAG has a significantly lower level of 16:3 acyl groups, which are predominant species in *A. thaliana* MGDG, indicating DAG produced by GGGT is used in TAG biosynthesis.

The role of *sfr2*-like genes in algae is yet to be determined. The chloroplast membrane of *C. reinhardtii* lacks GGGT (Mendiola-Morgenthaler et al, 1985) and genes with significant sequence similarity to GGGT/*sfr2*-like genes have not been found in the complete genome sequence (Fourrier et al, 2008).

#### 1.1.4.3 Phospholipid:diacylglycerol acyltransferase

In plants and yeasts an acyl-CoA independent pathway exists for TAG biosynthesis (Dahlqvist et al, 2000; Oelkers et al, 2000; Stahl et al, 2004). This pathway is catalysed by phospholipid:diacylglycerol acyltransferase (PDAT), using phospholipid as an acyl donor and DAG as an acyl acceptor. In *S. cerevisiae* overexpression of the gene encoding PDAT resulted in a considerable increase in TAG during the logarithmic growth phase (Dahlqvist et al, 2000), suggesting a major role for PDAT in TAG biosynthesis during this growth phase. However, overexpression and knockout experiments in *A. thaliana* did not have any effect on lipid biosynthesis in seedlings (Stahl et al, 2004; Mhakske et al, 2005), suggesting that PDAT serves a different role in plants. Further experiments revealed that PDAT in *A. thaliana* has overlapping functions with DGAT1 for TAG biosynthesis (Zhang et al, 2009).

A TAG-synthesising PDAT enzyme has been cloned and characterised in *C. reinhardtii* (Boyle et al, 2012; Yoon et al, 2012). This PDAT shares homology with previously characterised plant and yeast PDATs as well as mammalian lecithin:cholesterol acyltransferases (LCAT) (Yoon et al, 2012). A knockout of the PDAT in *C. reinhardtii* by insertional mutagenesis resulted in TAG being reduced by 25% under nitrogen deprivation (Boyle et al, 2012). Further experiments on this PDAT, using micro-RNA-mediated gene silencing to produce knock-down mutants, led to increased accumulation of phosphoglycerolipids, such as phosphatidylglycerol (PG) and MGDG sulfoquinovosyldiacylglycerol (SQDG) (Yoon et al, 2012). These results suggest that PDAT in *C. reinhardtii* utilises these phosphoglycerolipids as substrates for TAG biosynthesis. *In vitro* and *in vivo* enzyme assays have shown that PDAT contributes to TAG synthesis in *C. reinhardtii* via two separate pathways. The first is through the transacylation of DAG with acyl groups from a number of phospholipid species and MGDG; and secondly through DAG:DAG transacylation, producing TAG and monoacylglycerol (Yoon et al., 2012).

#### 1.1.4.4 Conversion of starch to TAG

Another common energy storage compound commonly occurring in plants and algae is starch. Starch is accumulated as the principle storage compound by many algae, particularly the chlorophyta (green algae), especially under stress conditions. Under nitrogen deprivation conditions in *C. reinhardtii* starch content can reach up to 45% of dry cell weight (Li et al, 2010).

The first committed step of starch synthesis is the conversion of glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate, catalysed by ADP-glucose pyrophosphorylase (AGPase) (Ball and Morell, 2003).

Starch synthesis shares common precursors with lipid synthesis, thereby interconnecting these two pathways. The possible interaction between the two pathways has been investigated in *A. thaliana* seeds and *B. napus* embryos (Kang and Rawsthorne, 1994; Ruuska et al, 2002). These studies found starch metabolism was most active before oil accumulation and that there was transient accumulation of starch during this period. Other studies have found that in plant embryos and seeds where starch synthesis was blocked or impaired there was a lower accumulation of TAG (Periappuram et al, 2000; Vigeolas et al, 2004), demonstrating a clear link between starch and TAG synthesis. This interaction has also been observed in the diatom *C. cryptica*, with the conversion of carbohydrates to lipids under silica stress conditions (Roessler, 1988). The mechanisms responsible for this are unknown. Inhibition of AGPase in *C. reinhardtii* resulted in a starchless and TAG-excess phenotype (Li et

al, 2010), indicating that starch and lipid synthetic pathways may compete for carbon precursors.

### 1.1.5 Lipid Body Biogenesis

In eukaryotic microalgae the high levels of TAG that are synthesised are sequestered into subcellular organelles, lipid bodies (LBs), under stress conditions. Cytosolic LBs are the major TAG deposits in oleaginous microalgae. LBs can also form on the inner chloroplast envelope in the thylakoid membranes. These LBs are smaller than cytosolic LBs and are known as plastoglobules (Austin et al, 2006).

LBs are distinct from other subcellular organelles in that they are separated from the aqueous cellular environment by a protein-bearing monolayer, rather than a membrane bilayer (Murphy and Vance, 1999; Murphy, 2001). This monolayer encircles a hydrophobic neutral lipid core. The biochemical composition of LBs varies among species, but they do share the same major components – neutral lipids (80% w/w), e.g. TAG, low levels of phospholipids (1 – 5% w/w) and proteins (1 – 5%) (Vechtel et al, 1992; Zweytick et al, 2000).

The monolayer membrane of LBs consists of a variety of glycerolipid species. In *C. reinhardtii* LBs, 1, 2-diacylglyceryl-3-O-4'-(N, N, N-trimethylhomoserine) (DGTS) is the most abundant glycerolipid, with the presence of PE, PG, SQDG, and digalactosylglycerols (DGDG) as the minor components of the monolayer membrane (Wang et al., 2009). This is in contrast with mammalian LBs where PC is the most abundant phospholipid (~50% of total membrane lipids), followed by phosphatidylethanolamine (PE) (~30–40% of total membrane lipids). In higher plant LBs, again, PC is the major phospholipid, and minor components include phosphatidylserine (PS), PE, and phosphatidylinositol (PI). The phospholipids of yeast LBs consist of approximately equal amounts of PC, PE, and PI (Zweytick et al., 2000).

LB neutral lipids predominantly consist of TAG, DAG, free fatty acids and sterol esters. In microalgae and higher plants LBs comprise mainly of TAG (more than 90%) (Zweytick et al, 2000; Wang et al, 2009), whereas yeast LBs contain almost 50% TAG (Walther and Farese, 2009) and mammalian LBs contain large amounts of free fatty acids and esterified cholesterol instead of TAG (Zweytick et al, 2000).

The structural and biochemical compositions of algal LBs are believed to be different among different classes of microalgae (Vieler et al, 2012).

#### 1.1.5.1 LB biogenesis in eukaryotic microalgae

In microalgae, LB accumulation seems to be induced under environmental stress conditions. Recent studies have suggested the involvement of plastids in LB formation in *C. reinhardtii* (Fan et al, 2011; Goodson et al. 2011); along with the observation of LBs in both the plastid and cytosol of a starch-less mutant of *C. reinhardtii* (Fan et al. 2011). Plastidial participation in LB biogenesis is further supported by a likely plastidial location of TAG-synthesising enzymes in *C. reinhardtii*, as determined by an algorithm designed for predicting microalgal protein targeting signals (Tardif et al. 2012). Observations of LBs physically connected to the chloroplast envelope membranes in *C. reinhardtii* have been reported (Li et al, 2013), further supporting plastidial involvement in LB biogenesis in microalgae. Li and colleagues also refer to additional transmission electron microscope studies of *C. reinhardtii* where they observed lipid microdomains in the chloroplast envelope membranes, and suggest that chloroplast-associated LBs may be formed from the blistering of the leaflets of the chloroplast envelope membranes.

#### 1.1.5.2 Functions of LBs in microalgae

In microalgae, LBs are generated in response to particular cellular needs. The number of lipid droplets per cell varies depending on cell type, developmental stages and growth conditions. LBs have long been considered to just function as lipid storage organelles, but increasing knowledge of LBs has revealed a more complex role in microalgal cells, particularly under stress conditions.

In *C.reinhardtii*, a number of LB proteins are involved in lipid metabolism, including enzymes active in fatty acid metabolism, glycerolipid biosynthesis, sterol biosynthesis, and lipolysis. GPAT, LPAAT and DGAT enzymes involved in acyl-CoA-dependent TAG biosynthesis have also been found in LBs, as well as acyl-CoA-independent PDAT, suggesting a role for PDAT in an alternative route to TAG synthesis in LBs (Moellering and Benning, 2010; Nguyen et al, 2011). These studies also revealed several lipases are present in LBs in *C. reinhardtii*.

TAGs contain twice as much energy than starch or protein per weight unit. Storage of TAG is therefore the most efficient and compact way to sequester energy. In microalgae, under stress and nutrient-deplete conditions, cells store extra carbon as TAGs, for long-term usage and survival (Hu et al, 2008).

TAG accumulation and storage in LBs could also serve as a temporary resevoir of acyl chains, as suggested by the appearance of LBs under iron starvation conditions, where no net

synthesis of fatty acids occurred (Urzica et al, 2013). Under adverse conditions, e.g. extreme cold and osmotic stress, changes in bilayer structures are compensated for by the removal of membrane lipids. These released membrane lipids provide free acyl chains which can be incorporated into TAG in LBs. Upon the return of favourable environmental conditions, hydrolysis of TAG can release these acyl chains to be utilised in the synthesis and assembly of membrane lipids. This mechanism allows the fast shuttling of free acyl chains to membrane lipid synthesis and vice versa, bypassing the need for *de novo* fatty acid synthesis under adverse conditions (Goold et al, 2014).

Microalgal LBs may also have a number of diverse functions and therefore a more complex role in algal cells. This is based on the identification of a large array of proteins in the LB proteome; proteomic analysis of LBs isolated from *C. reinhardtii* has revealed large amounts of proteins involved in lipid metabolism, intracellular trafficking, posttranslational modification, and protein turnover (Nguyen et al, 2005; Moellering and Benning, 2010). These results suggest a role for LBs in cell signalling, protective roles, as cargo for compound delivery to their subcellular location, as a reservoir for some compounds, in the catabolism of glycerolipids and sterol esters, etc. However, confirmation of algal LBs in these roles is yet to be confirmed experimentally and this process is slow due to the lack of mutants in LB biogenesis and metabolism.

#### 1.1.5.3 LB interactions with other organelles

Observations of LBs interacting with other cellular organelles (e.g. mitochondria and ER) (Cushman, 1970; Novikoff et al, 1980; Goodson et al, 2011), as well as the presence of lipid transport proteins known to be membrane contact site proteins (Holthuis and Levine, 2005; Goodson et al, 2011; Pidoux et al, 2011), suggest that lipid metabolism, storage and transport is a complex process involving interactions between multiple cellular organelles and LBs.

Knowledge about the structure, function, and biogenesis of algal LBs is crucial for understanding lipid homeostasis and cell metabolism. Identification of LB-associated proteins in model organisms suggests that algal LBs are not static storage organelles, but are involved in multiple cellular functions. These LB proteins could be promising targets for genetic engineering for the rational manipulation of TAG production in oleaginous microalgae.

### 1.1.6 Physiological Role of TAG in Microalgae

Recent research is providing growing evidence that TAG does not just serve as a carbon and energy reserve, but that it may also be important in lipid homeostasis. Observations in yeast postulate a role for TAG in the provision of metabolites required for cell cycle progression and sporulation (Kohlwein, 2010).

In algae, TAG biosynthesis and LB biogenesis may be functionally coupled with photosynthesis. During favourable photosynthetic conditions, the majority of electrons generated from the electron transport chain are utilised for the assimilation of inorganic carbon, nitrogen and sulphur, with the rest used for the oxidation of oxygen to reactive oxygen species (ROS) and for the synthesis of TAG. Under stress conditions, such as nitrogen deprivation and extremes of temperature and light, there may be an excess of electrons which would result in the acceleration of the formation of ROS, resulting in cellular membrane and protein damage, as well as the inhibition of photosynthesis. TAG synthesis may therefore be upregulated in order to avoid this (Hu et al, 2008). This theory has been supported recently with experimental evidence that TAG synthesis serves as an electron and energy sink, protecting the cell from excess ROS by relieving the over-reduced photosynthetic electron transport chain under stress (Li et al, 2012). Li and colleagues characterised a *C. reinhardtii* mutant with a deficiency in galactoglycerolipid lipase activity. Under nitrogen deprivation conditions the mutant exhibited accelerated cell death. Cell viability was restored by inhibiting the photosynthetic electron transport chain.

TAG biosynthetic pathways may also be coordinated with those synthesising carotenoids (Rabbani et al, 1998; Zhekisheva et al, 2002; Chen, 2007). The carotenoid astaxanthin (β-carotene) is sequestered to cytosolic or platidial LBs. The carotenoid biosynthetic pathways also utilise electrons and oxygen produced from photosynthesis in the chloroplasts. Carotenoid biosynthesis may therefore also protect cells from excess ROS by synthesising oxygen-rich astaxanthin esters. LBs rich in astaxanthin are generally localised to the periphery of algal cells and may therefore also serve a role in photoprotection, protecting the chloroplast from excessive light (Hagen et al, 1994).

## 1.2 OMEGA-3 FATTY ACIDS

The genetic manipulation of microalgae to enhance their production of omega-3 fatty acids and the subsequent accumulation of omega-3s in TAG relies on full understanding of the above pathways, processes and mechanisms. Genetically engineered microalgae could prove to be a valuable sustainable source of essential omega-3s which have numerous benefits in treating human diseases such as cardiovascular disease (CVD) (Masson et al, 2007; Saravanan et al, 2010), neurological disorders (Freeman et al, 2006), obesity (Buckley & Howe, 2009) and cancers (Siddiqui et al, 2008). Furthermore, the omega-3 fatty acid docosahexaenoic acid (DHA) has been shown to be a crucial factor in brain and eye development (Innis, 2008).

### 1.2.1 Physiological Importance of Omega-3 Fatty Acids in Humans

Omega-3 and omega-6 fatty acids are metabolically distinct and have opposing physiological functions. Omega-3 fatty acids play a vital role in human health. Humans, like most other animals, are unable to synthesise the omega-3 fatty acids *de novo* (Nakamura and Nara, 2008). Omega-3 fatty acids are therefore termed as “essential” fatty acids since they must be obtained through dietary intake. They are vital functional components of the cell membrane, regulating structure, fluidity and permeability; but not just in a structural capacity, they also have importance in human metabolism as precursors of bioactive molecules such as eicosanoids, metabolites which help to regulate inflammation, plaque aggregation, immune-reactivity and vasoconstriction and dilation (Funk, 2001 and Tapiero, 2002). ARA and DHA have been shown as crucial in neonatal health and development, with particular regards to brain development and the acquirement of ocular vision (Innis, 2008). As a result, ARA and DHA have been recommended to be included in infant formula milks (Agostoni, 2008). EPA and DHA have been recognised through clinical studies as having protective effects against metabolic syndrome, including the related obesity and type-2 diabetes disease states, cancers, atherosclerosis, cardiovascular disease, dementia, childhood attention-deficit hyperactivity disorder (ADHD) and mental illnesses, particularly depression. Omega-3 fatty acids have also been shown to confer beneficial effects to the immune system, reproductive system, in the resolution of inflammation and in skin barrier function.

#### 1.2.1.1 Omega-3 fatty acids and acquisition of ocular vision and brain development in infants

Sufficient levels of DHA are important for maintaining neonatal health and brain development. DHA remains an important factor in development throughout infancy. DHA is an integral component of retinal phospholipids and brain grey matter. A low intake of omega-3 fatty acids results in decreased levels of DHA and increased levels of omega-6 fatty acids. This can lead to impaired neurogenesis and development of the retina and brain, causing sub-optimal altered cognitive and visual function. This has highlighted the importance of a sufficient intake of omega-3 fatty acids during pregnancy, if breast-feeding and throughout the child's infancy. It has been recommended that infant formula milks contain adequate levels of DHA to ensure infants obtain adequate levels of nutrition for optimal development (Innis, 2008; Agostoni, 2008).

#### 1.2.1.2 Omega-3 fatty acids and metabolic syndrome, obesity and type-2 diabetes

Through their effects on lipid metabolism, omega-3 fatty acids have been postulated as having potential benefits in the alleviation and possible prevention of obesity, metabolic syndrome and type-2 diabetes (Buckley and Howe, 2009; Flachs et al, 2006; Ruxton et al, 2004). By conferring a suppressive effect on lipoprotein lipase, reducing its ability to hydrolyse triglycerides, the uptake of lipids is lowered, thus reducing body fat accumulation. Buckley and Howe (2009) reported that increased consumption of EPA and DHA led to protection against the development of obesity in animals exposed to an obesogenic diet. They also observed reductions in body fat in animals that were already obese. Longer term studies need to be undertaken to determine if the effect is the same in humans, however, the evidence so far, including observational studies (Ruxton et al, 2004), is compelling.

#### 1.2.1.3 Omega-3 fatty acids and cardiovascular disease

Consumption of omega-3 fatty acids contributes to reducing a person's risk of suffering from cardiovascular disease (CVD) through the lowering of blood pressure and heart rate. Furthermore, arrhythmias, inflammation, hypertension and atherothrombosis, which contribute to cardiovascular disease, are reduced due to the anti-inflammatory, antithrombotic, and antiarrhythmic properties of n-3 FAs. In addition, studies have shown that people with diets high in omega-3 consumption (i.e. those who consume a lot of fresh fish) have a lower risk of coronary artery disease. The evidence is compelling for a cardioprotective effect of omega-3 fatty acids (Covington, 2004; Marik and Varon, 2009; Masson et al, 2007).

#### 1.2.1.4 Omega-3 fatty acids and cancer

Observational studies have reported lower incidences of cancer in populations whose diet is high in omega-3 consumption. It has been postulated that DHA confers a protective effect on healthy cells against apoptosis due to the high levels of DHA found here; however, in unhealthy cells, DHA is found at much lower levels and induces apoptosis. It has been speculated that DHA could have potential benefits in increasing the effectiveness of chemotherapy whilst minimising side effects associated with this treatment. Furthermore, evidence in recent studies has demonstrated that omega-3 fatty acids can inhibit the proliferation of cancer cells, and also have anti-inflammatory and anti-carcinogenic effects (Daniel et al, 2009; Dimri et al, 2010). In addition, omega-3 fatty acids are shown to negate the pro-inflammatory effects of omega-6 fatty acids, further highlighting the importance for a good balance between these fatty acid classes (Hardman, 2002; MacLean et al, 2006; Siddiqui et al, 2008).

#### 1.2.1.5 Omega-3 fatty acids and inflammatory and autoimmune diseases

EPA and DHA possess potent immunomodulatory activities, either through the types and amounts of eicosanoids, or through their effects on intracellular signalling pathways, transcription factor activity and gene expression (Ruxton et al, 2004; Simopoulos, 2002). Those suffering from autoimmune diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease respond positively to EPA and DHA supplementation as a result of the reduction of cytokine levels, which are normally elevated in these patients. Additionally, studies in animals and humans have shown that omega-3 fatty acids suppress cell mediated immune responses.

#### 1.2.1.6 Omega-3 fatty acids and neurological disease and mental health

Neurodegenerative diseases such as Alzheimer's have been shown to be coupled with low levels of DHA in blood serum and brain membranes (Kim, 2008). Deficiencies in DHA and EPA in healthy individuals have also been associated with depression (Freeman et al, 2006; Ali et al, 2009); animal studies have demonstrated low levels of these omega-3 fatty acids led to decreased levels of the neurotransmitters serotonin and dopamine, which are important in regulating mood. Further studies have shown that EPA and DHA supplementation of children with juvenile bipolar disorder led to decreased incidences of mania and depression (Clayton et al, 2009). It is believed that omega-3 fatty acids have a similar action on signal transduction pathways as the drugs currently used to treat bipolar disorder; however, they have the advantage that they lack the side effects of these drugs and are not toxic.

### 1.2.2 Sources of Omega-3

The benefits of omega-3 PUFAs, especially EPA and DHA, are well established. Omega-3 PUFAs are synthesised primarily in the marine environment, and accumulate in the aquatic food web through consumption (Williams, 2006) (Figure 4). Marine microbes, particularly diatoms and microalgae, are the primary producers of these essential fatty acids, which are vital for human health but cannot be synthesised *de novo* (Nakamura et al, 2004). Although vegetable oils are also nutritionally valuable, they do not deliver the same health benefits as fish oils, thus making fish oils a valuable commodity with many different applications; including use as dietary supplements.

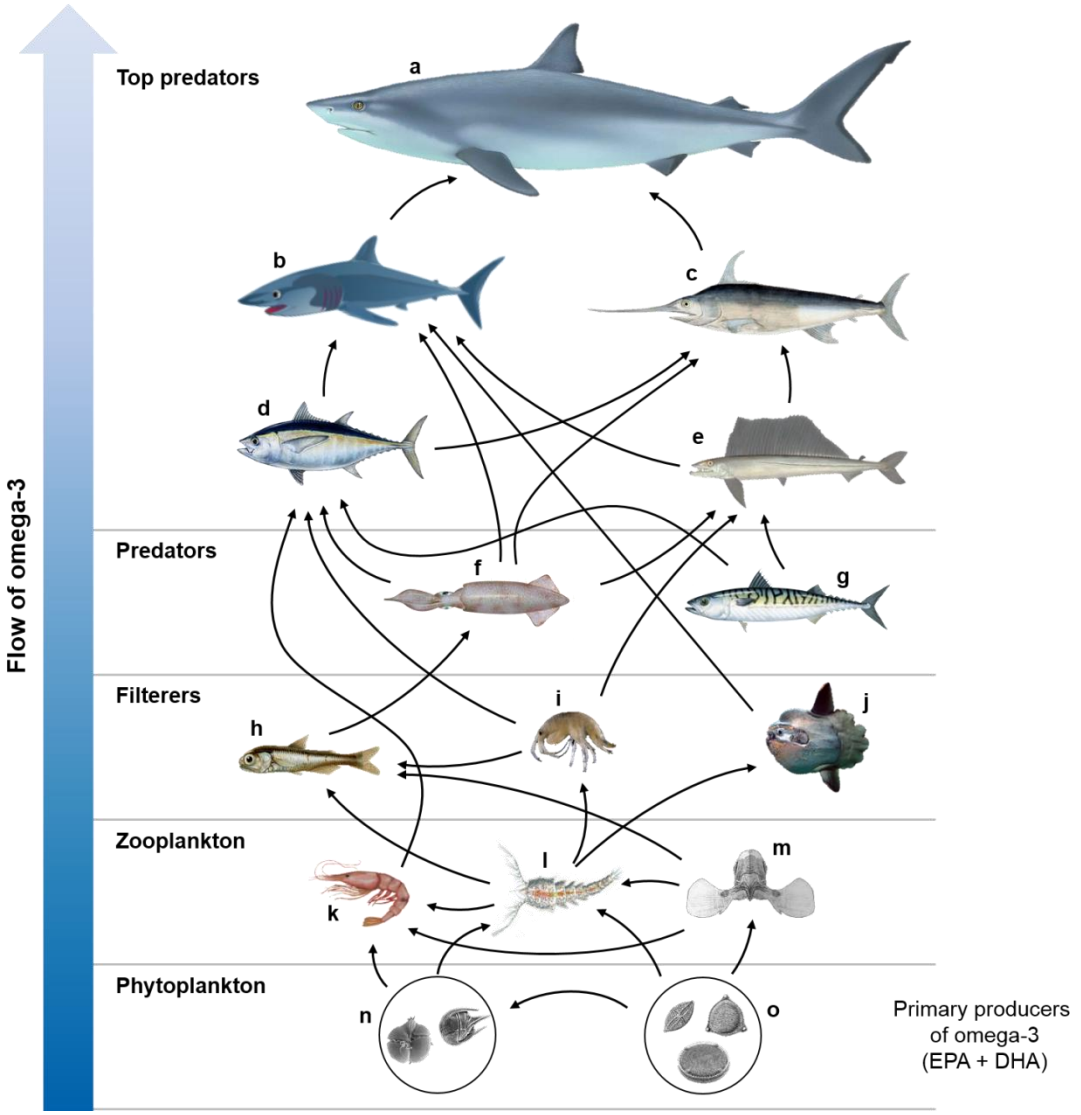



**Figure 4:** The flow of omega-3 through the aquatic food web. A – large sharks; b – smaller sharks; c – marlin; d – tuna; e – lancet fish; f – squid; g – mackerel; h – lantern fish; i – amphipods; j – ocean sunfish; k – shrimp; l – copepods; m – pteropods; n – dinoflagellates; o – diatoms.

#### 1.2.2.1 Current Sources

##### 1.2.2.1.1 Fish

The current major source of omega-3 fatty acids is fish oils. Fish oils that are rich in omega-3 are derived from the tissue of oily fish (mackerel, sardine, salmon and herring) which have accumulated these essential fatty acids through consumption. Fisheries process wild or farmed fish, resulting in a variation in end fish oil content, as well as differing sustainability issues which will be discussed below. Fish processing plants that extract the oil can also produce fishmeal and stickwater, a viscous liquor-like by-product which can be concentrated and used in fish and animal feeds as a source of amino acids and vitamins. Fishmeal is also a valuable commodity in the fishing industry, and is often produced along with fish oil. Since not all methods that extract oil give rise to fishmeal and stickwater, the method utilised will be determined by the desired end products. Techniques to extract oil include:

1) Wet reduction (outlined below);
2) Cold extraction (fish ground and centrifuged to separate oil; low yields (Rubio-Rodríguez et al, 2012));
3) Enzyme extraction (use of proteolytic enzymes; typically used for fish or offal with low oil content (Gbogouri et al, 2006));
4) Solvent extraction (solvents used to remove water, fat and compounds responsible for "fishy taste" (Moffat et al, 1993));
5) Supercritical fluid extraction (supercritical $CO_2$ used to extract high yields of omega-3 rich oil (Rubio-Rodríguez et al, 2008)).

Most of the world's supply of fish oil is extracted using the wet reduction method (FAO, 1986). This involves:

1) Cooking at high temperatures to coagulate protein and liberate bound water and oil, followed by:
2) Separation through pressing of the coagulate to yield a "presscake", which contains oil-free dry matter and oil, and a liquid "press liquor" phase (containing water, oil and dissolved proteins, vitamins and minerals).
3) Oil is obtained from the press liquor through centrifugation. The remaining stickwater is concentrated and added to the presscake before being dried and milled to be used as feed.

However, all the techniques mentioned above present drawbacks. The heating process in wet reduction can affect the quality of omega-3 PUFAs (Gbogouri et al, 2006). Cold extraction, enzyme extraction and solvent extraction can lead to oxidation of the PUFAs, which, again, is undesirable. Recent advances in supercritical fluid extraction (Rubio-Rodríguez et al, 2012) aim to overcome these issues, however, its widespread adoption may be hampered due to the high costs involved.

As supplements fish oils have undesirable flavours and odours, as well as having varying and unguaranteed quality (*Consumer* Reports: "Fish Oil Pills vs. Claims," January 2012). Increasing global demand for omega-3 fatty acids has put a strain on the fishing industry (Pauly et al, 2005); in conjunction with diminishing wild fish stocks and widespread pollution of the marine environment (Garcia and Rosenburg 2010) this highlights the growing need for alternative, sustainable sources of these essential omega-3 fatty acids, particularly EPA and DHA.

One strategy already implemented long-term in an attempt to tackle the overfishing of wild populations is the establishment of fish farms. The aquaculture industry is one of the fastest growing food production industries in the world, with the majority of the output being used for direct human consumption (Klinger et al, 2012 and FAO, 2014). However, the proportion of the world fisheries' production that is then processed into fish oils is declining due to the cost, environmental phenomena, such as El Niño, and increasing pressure to improve sustainability, resulting in strict management measures that limit the volume of oil that can be produced (FAO, 2014). Furthermore, intensification of production means that it is also not an environmentally sustainable industry (FAO, 2014). This issue is further compounded by the fact that in order to achieve the desired content of EPA and DHA in the end product, farmed fish must have their diets supplemented with additional fatty acids so that they are able to synthesise and accumulate the long-chain PUFAs (Ruiz-Lopez et al, 2012). This supplementation can be through formulated fish feeds which include plant-based additives (Aliyu-Paiko et al, 2011), marine microorganisms (Ruiz-Lopez et al, 2012), or fishmeal which consists of caught wild fish (FAO, 2014). Reliance on the aquaculture industry as a source of omega-3 PUFAs is therefore not viable in the long-term.

#### 1.2.2.1.2 Krill

The small crustacean krill is another existing source of omega-3 rich oil. Oil derived from krill is also processed in fisheries using similar methods described above. Krill oil is particularly

omega-3 rich and has the added health benefits of containing high levels of antioxidants, such as astaxanthin (Nicol et al, 2012).

Krill are found in all the oceans, but are most abundant in the Antarctic Ocean (Atkinson et al, 2009), where they are commercially captured. Since krill are a swarming species, their capture is made relatively straightforward, with a high yield of biomass being obtained from dense swarms (Gigliotti et al, 2011). However, measures have been put in place to limit krill catches and ensure long-term sustainability due to their important role in the marine ecosystem (Constable, 2011). This again addresses the need for alternative sustainable sources of omega-3.

#### 1.2.2.1.3 Single cell oils

It would seem logical that, since marine microalgae are the primary source of omega-3 PUFAs, the obvious method of production would be the direct cultivation of EPA and DHA-rich microalgae. Oleaginous microorganisms, i.e. those which accumulate oil, can be cultured directly on an industrial scale as sources of oils and fats. These products are collectively known as single cell oils, or SCOs. Unlike fish, whose fatty acid profile depends on their diet, oleaginous microorganisms, like higher plants, only synthesise fatty acids which they have the genetic capability to do so. They have a relatively simple fatty acid profile which can vary between the different species, thus, a specific microorganism can be selected to produce a "designer oil" with a precise fatty acid composition (Kyle, 1992). Such designer oils are superior to fish oils because they can be of a higher quality and consist of only one bioactive fatty acid, unlike fish oils.

Primary producers of omega-3s, which accumulate these PUFAs as triacyglycerols, are cultivated auxotrophically or heterotrophically in bioreactors. Such organisms include microalgae, some fungi and yeasts (Barclay et al, 1994). Large-scale culturing of aquatic microbes that produce omega-3 fatty acids is already undertaken for high value applications, such as for use in infant formula milks (discussed below) or nutraceuticals, but these fermentation methods are difficult to scale-up and are a particularly costly method of production (Agostoni, 2008; Lee, 2001).

Harrington and Holz first reported the high content of DHA in the non-photosynthetic dinoflagellate *Crypthecodinium cohnii* (previously *Gyrodinium cohnii*) in 1968, as well as a full characterisation of its fatty acid methyl esters (FAMEs). DHA is the only PUFA accumulated to levels greater than 1% by *C. cohnii* and it has a basic FAMEs profile (Harrington and Holz,

1968). Almost 25 years later *C. cohnii* was utilised as the first commercial microalgal source of single cell derived DHA-rich oil, known as DHASCO, developed and marketed by Martek Biosciences Corporation (Kyle, 1992; Kyle, 1996; Kyle, 2001). DHASCO was combined with another designer oil rich in ARA to produce Formulaid, a supplement for use in infant formula milks (Kyle, 1992). *C. cohnii* was chosen not only because of its ability to accumulate high levels of DHA (up to 43% of total oil in later optimisations by Martek (Kyle, 2001)), but also because it can be grown heterotrophically and could thus be cultured in a traditional fermentor system, therefore overcoming the prohibitive costs that were then associated with the scale-up culture of photosynthetic organisms (Kyle, 1996).

The production levels of PUFAs and costs associated with oils from photosynthetic microalgae have been investigated. Molina Grima et al (1996) produced a highly concentrated EPA fraction from the diatom *Phaeodactylum tricornutum*. With a daily productivity of almost 2.5g using their three-step process, the estimated production costs were calculated at $188 per day, cheaper than obtaining EPA from cod liver oil. However, they did caution that the figures may not translate so linearly when the system that they researched is scaled up further. They concluded that although their results looked promising, further research would have to be undertaken, with particular regards to the hydrodynamics of the system as well as the mass transfer coefficient, in order to make such generalised correlations between an experimental and industrial system.

Scale up and the cost of mass production are still the main barriers to overcome with regards to single cell and algal oils. The focus of research in this area is now on optimising this process and reducing the cost of production (Harwood and Guschina, 2009), as well as alternative strategies for the production of omega-3 rich oils from microalgal species, which will be discussed in more detail in the following section.

#### 1.2.2.2 Alternative Sources

##### 1.2.2.2.1 Transgenic plants

One alternative source of omega-3 oils, which has been explored with some promising results in recent years, is the generation of transgenic oilseed crops expressing genes for the production and accumulation of valuable omega-3 PUFAs (Ruiz-Lopez et al, 2013; Sayanova et al, 2011 and Venegas-Calerón et al, 2010).

The first successfully genetically modified plants were reported in 1983 by four independent groups (Herrera-Estrella et al, 1983; Bevan et al, 1983; Fraley et al, 1983; Murai et al, 1983).

Herrera-Estrella et al described the successful transformation of tobacco plants with bacterial genes conferring kanamycin and methotrexate resistance; Bevan et al reported kanamycin-resistant *Nicotiana plumbaginifolia* plants; Fraley et al produced kanamycin-resistant petunia plants and Murai et al successfully transformed sunflower plants with genes from bean plants. This collective work paved the way for a new approach to breeding plants with novel traits and new plant genetic engineering technologies.

The race to produce the first transgenic plant in 1983 began with the discovery of the Tumour-inducing plasmid (Ti-plasmid) from *Agrobacterium tumefaciens* (Schell and Montagu, 1977). This was followed by investigations into the specific bacterial DNA transfer mechanisms that had been observed (Chilton et al, 1977; De Picker et al, 1978 and Zambryski et al, 1980). The next challenge was to convert the Ti-plasmid into a gene expression vector; the first successful non-oncogenic vector was reported in 1982 (Zambryski et al, 1982). The following year saw the first multipurpose plant vector system (Hoekema et al, 1983), which is the system still in use today and known more commonly as the T-DNA binary system, or the binary vector system. All of the transgenic plants successfully produced in 1983 were the result of the *A. tumefaciens* method. The discovery and development of this transformation method through the concerted efforts of hundreds of scientists revolutionised plant molecular biology and spurred scores of scientists to utilise this new technology to overcome multiple agricultural challenges, to the point where millions of hectares of land are now used to cultivate genetically modified crops (James, 2012).

Since 1983 there have been some particularly notable applications of the use of genetic engineering in plants:

1. "Gain of function" species with novel traits through the introduction of transgenes, including:
    a. **Nutritionally improved species** – e.g. the famous "Golden Rice" project which produced a genetically modified *Oryza sativa* rice species capable of producing beta-carotene, the precursor to vitamin A. The goal was to combat vitamin A deficiency by producing fortified rice that could be grown in areas with a shortage of dietary vitamin A, areas in which rice is a staple food (Ye et al, 2000);
    b. **Insect resistant species** – e.g. Bt maize crops engineered to release toxins originating from the soil bacterium *Bacillus thuringiensis* (Bt) which protect the plant from the European corn borer (Hellmich, 2012);

c. **Drought resistant species** – e.g. Monsanto's DroughtGard genetically modified maize crop, the first to receive US marketing approval (Eisenstein, 2013);

2. "Loss of function" species containing interfering or anti-sense RNAs to suppress a native trait. For example the "Flavr savr" tomato by Calgene, the first genetically modified food to be granted permission for human consumption (Bruening and Lyons, 2000). These transgenic tomatoes were engineered with antisense RNA to suppress the production of polygalacturonase, an enzyme involved in the ripening process and whose suppression resulted in the fruit staying firmer for longer.

The advent of plant biotechnology saw rapid progress in genetic engineering, not only from introducing new phenotypes from one plant to another, but also in producing novel valuable products that are not naturally occurring in these organisms. Designer plant oils containing non-native fatty acids were one of the first of such valuable commodities that entered the market in the mid-nineties. Calgene Inc (Davis, CA) developed a canola oil with a high laurate content, commercially known as Laurical, the first genetically engineered plant oil to be successfully commercialised (Yuan and Knauf, 1997). Further understanding of lipid biosynthesis and storage in plants and the discovery of genes encoding enzymes responsible for fatty acid synthesis in other organisms, in tandem with the development of more sophisticated engineering techniques, greatly aided the field of research into designer oilseeds.

There are many end-use applications for the oils extracted from designer oilseeds, such as in the food industry due to improved nutritional qualities (Broun et al, 1999; Tucker, 2003; Robert, 2006), as lubricants (Jaworski and Cahoon, 2003), as a feedstock in the chemical industry (Singh et al, 2005), and even as a source of biofuels (Kinney and Clemente, 2005). The amenability to such a range of applications is in part due to the wide variation of fatty acids that higher plants naturally produce, as well as the ability to exploit this variation and introduce the ability to synthesise new or highly tailored oils through genetic engineering. Although the fatty acid profiles of the seed oils of higher plants share few commonalities (Napier, 2007), one trait that is shared is that in general the unusual fatty acids are synthesised in the developing seed tissues and accumulate as TAG. The level of accumulation is not often high enough to be amenable for industrial use. However, since seed oil variation seems to already be tolerated by the plant (plant membrane fatty acid composition is highly conserved (Ohlrogge, 1994)), it can be concluded that any changes in the seed oil

composition through genetic engineering are unlikely to negatively affect the plant physiology. Therefore, pathway engineering of higher plants to produce long-chain polyunsaturated acids is an attractive option. Furthermore, the aforementioned costs and complexities of industrial algal culture (see section 1.2.2.1.3) for PUFA production has lead to further interest in engineering plants for the production of omega-3 rich oils.

Higher plants lack the natural ability to synthesise long-chain PUFAs such as EPA and DHA, so significant efforts are required to establish the required pathways in plants. Some species are able to synthesise the Δ6-desaturation products GLA and SDA (Traitler, et al 1984; Ucciani, 1995; Sayanova and Napier, 1999). Δ6-desaturation is the first committed step in the PUFA biosynthetic pathway and early attempts at transgenic PUFA production in plants focused on this activity (Reddy and Thomas, 1996; Sayanova et al, 1997). Reddy and Thomas stably integrated a cyanobacterial Δ6-desaturase encoding gene into tobacco plants under the control of a strong constitutive promoter using the *A. tumafaciens* transformation method. They reported the low level accumulation of GLA and SDA. Higher accumulation, up to 20% of total fatty acids, was reported by Sayanova et al the following year in their transgenic tobacco plants transformed with constructs containing the cDNA of a borage Δ6-desaturase, again under the control of a constitutive promoter. In both of these experiments the GLA and SDA accumulated in the leaves of the transgenic plants. Later experiments using an embryo-specific promoter, which led to seed-specific expression, found that higher levels of GLA and SDA accumulation were possible (between 40 – 70% of total fatty acids) (Hong et al, 2002; Qiu et al, 2002; Sato et al, 2004).

The “alternative” Δ8-desaturase pathway (Sayanova and Napier, 2004) has also been reconstituted in plants, yielding more favourable results in terms of the level of accumulation – 6.6% ARA and 3% EPA of total fatty acids (Qi et al, 2004) – compared to those previously described at the time for the standard Δ6-pathway. Under the control of a constitutive promoter, three genes encoding the primary activities for the Δ8-pathway were sequentially introduced into *Arabidopsis thaliana* plants. The genes used were from diverse organisms; an *Isochrysis galbana* Δ9-elongase (Qi et al, 2002), a Δ8-desaturase from the protist *Euglena gracilis* (Sayanova and Napier, 2004) and a *Mortierella alpina* Δ5-desaturase (Michaelson et al, 1998). Although initial results appeared to be promising, later studies revealed inefficient transfer of the non-native fatty acids out of the acetyl-CoA pool (Sayanova et al, 2006), which could cause a bottleneck effect and therefore limit the accumulation of LC-PUFAs.

In order to produce the long-chain PUFAs EPA and DHA, three further enzymatic activities must be introduced into the plant; a Δ6-desaturase, Δ6-elongase and a Δ5-desaturase (and a fourth, a Δ4-desaturase, for the production of DHA). The first successful efforts at reconstituting these activities in plants for the production of ARA and EPA, in this case in linseed and tobacco, used genes encoding the Δ6 and Δ5-desaturases from the diatom *Phaeodactylum tricornutum*, and a Δ6-elongase-encoding gene from the moss *Physcomitrella patens* (Abbadi et al, 2004). Under seed-specific promoters, transgenic lines accumulated more than 25% SDA and GLA (of total fatty acids), 2.7% of ARA and only low levels of EPA at 1.6%. It was inferred from these results that a bottleneck was occurring at the elongation step. Although the results demonstrated that there was efficient Δ6-desaturation of LA and ALA, they revealed what would later be termed the "substrate dichotomy" (Napier et al, 2004). The low level of elongation was attributed to poor acyl-exchange of the GLA and SDA phospholipid-linked species to their acyl-CoA derivatives. Since desaturation occurred on the phospholipid-linked substrates and elongation on acyl-CoA substrates, this low incorporation of substrates into the acyl-CoA pool led to a rate-limiting flux between desaturation and subsequent elongation reactions. The "substrate dichotomy" arises due to the desaturase and elongase enzymes requiring these different substrate pools.

A similar approach was undertaken for the engineering of soybean using genes from the fungus *M. alpina* (Kinney et al, 2004). In an attempt to maximise the accumulation of omega-3 LC-PUFAs an ω-3 Δ17-desaturase from *Saprolegnia diclina* and an ω-3 Δ15-desaturase from Arabidopsis were co-expressed along with the Δ6 and Δ5-desaturases and Δ6-elongase in the soybean plants. These ω-3-desaturases convert omega-6 PUFA metabolites into their omega-3 counterparts. EPA was accumulated to 9.3% of total fatty acids in somatic embryos. When the *M. alpina* Δ6-desaturase was substituted with a Δ6-desaturase from *S. diclina*, EPA levels rose slightly to 13.3%. Plants derived from these embryos saw EPA accumulation in seeds of up to around 20%. With the addition of a Δ4-desaturase from *Schizochytrium aggregatum* and a Δ5-elongase from *Pavlova* sp., Kinney et al also observed the synthesis of DHA in seeds accumulating higher levels of EPA, along with the intermediate DPA. Unfortunately only low levels of DHA were observed, in the region of 2.0% – 3.3%. This work did, however, highlight the possibility of producing a more marine-like fatty acid composition in oilseeds, that is, a composition with an omega-3 bias and therefore with the desired profile in terms of nutritional value.

Despite the limited level of LC-PUFA accumulation and the complications surrounding the substrate dichotomy, researchers have not been discouraged from succeeding in this area. A

breakthrough occurred with the discovery of an acyl-CoA-dependent Δ6-desaturase from the microalgae *Ostreococcus tauri* (Domergue et al, 2005), which would allow the engineering of plants with targeted desaturase and elongase activities to the acyl-CoA pool, and thus circumventing the substrate dichotomy problem. Acyl-CoA-dependent desaturases insert a double bond into the CoA-activated fatty acid from the extraplastidial acyl-CoA pool, a markedly different reaction to the phospholipid-linked desaturases which require the fatty acid to be attached to PC or PE. The *O. tauri* Δ6-desaturase was found to desaturate ω3 and ω6 substrates with similar efficiencies when expressed in yeast (Domergue et al, 2005). Other acyl-CoA-dependent desaturases discovered more recently, however, have been found to be less promiscuous and have highly specific ω3 activity (Hoffman et al, 2008; Petrie et al, 2009; Petrie et al, 2010; Vaezi et al, 2013), demonstrating the strong preference of some acyl-CoA-dependent desaturases for ω3 substrates. The utilisation of acyl-CoA dependent substrates in transgenic plants resulted positively in a significant reduction in the levels of biosynthetic intermediates, notably GLA. Unfortunately, only low levels of accumulation of desirable target LC-PUFAs EPA and DHA have been observed (Hoffmann et al, 2008; Robert et al, 2005; Sayanova et al, 2012). Possible explanations for these observations could be that there are further, as yet unidentified, metabolic bottlenecks, or that the utilisation of non-optimised cDNA sequences results in poor expression in the transgenic host.

This further complication of diminished amounts of omega-3 rich oil that can be harvested from the transgenic crops, compared to natural producers, has recently been tackled with promising results. Ruiz-Lopez et al 2013 successfully demonstrated accumulation of EPA and DHA in the transgenic oilseed crop *Camelina sativa* to levels comparable to that of fish oil. Vectors containing cassettes expressing heterologous genes were designed for optimum EPA and DHA synthesis in *C. sativa*. Five genes were required for the EPA construct and seven for the DHA construct, all codon-optimised for *C. sativa* and under the control of seed specific promoters. Synthesis of EPA and DHA was efficiently directed to the seed oil and little accumulation of undesirable intermediate fatty acids was observed. Mean levels of 24% EPA accumulation were observed in the EPA iteration of transgenic plants, with maximum levels of up to 31% EPA. In the DHA iteration mean levels of EPA and DHA were 11% and 8% respectively, with maximum accumulation of 12% EPA and 14% DHA (Ruiz-Lopez et al, 2013). These results represent a promising terrestrial source of omega-3 PUFAs, should it be proven viable by further large scale investigations and field trials currently taking place (Rothamsted Research, 2014). However, there are still significant barriers, with regards to commercialisation, against making this a successful endeavour (Venegas-Calerón et al 2010).

Rigorous procedures for regulatory approval, that are costly in both time and money, are one such example (Kalaitzandonakes et al, 2007). Furthermore, such regulatory processes can vary widely between countries, with commercial efforts more likely to only be undertaken in countries where regulatory approval is more straightforward (Dunwell, 2014). Public perception can also play a critical role in the success of the commercialisation of a GM crop. Although plant-related GM applications were found to be more acceptable in a study of public perception of GM (Frewer et al, 2013), the end-product is likely to have an even greater impact. For example, were the omega-3-rich oil obtained from transgenic plants to be used for direct human consumption this is likely to be deemed less acceptable than the oil being used as aquaculture or animal feed and therefore for indirect human consumption. Given the disparity between political and commercial attitudes to GM crops in Europe and the US (Frewer et al, 2013; Dunwell, 2014), and the strong opposition to GM in Europe (Fresco, 2013), it is unlikely that the commercialisation of designer oilseeds will be an easy path. However, given the strong need for more sustainable sources of LC-PUFAs, transgenic plants are, to date, one of the most promising alternative options.

#### 1.2.2.2.2 Transgenic marine microalgae

As discussed previously, marine microalgae and diatoms are the primary producers of valuable omega-3 fatty acids and therefore a valid pursuit is to discover methods by which an efficient process can be found to obtain favourable amounts of omega-3-rich algal-derived oil. One way to overcome the biological barriers in producing enough oil of the desired composition is to metabolically engineer strains to increase their production and accumulation of EPA and DHA, and to direct their incorporation into TAG. At this time there isn't a naturally occurring species that is able to accumulate both EPA and DHA to high levels (Martins et al, 2013), so the targeted improvement of EPA and DHA accumulation in a selected species is an attractive and viable option.

Under certain naturally occurring conditions the level of synthesis of fatty acids and accumulation of lipids in microalgae can increase. Such conditions include nutrient deficiency (Pal et al, 2011), extremes in temperature (Van Wagenen et al, 2012), changes in light exposure (Liang et al, 2006), variations in salinity (Takagi, 2006) and nitrogen deficiency (Tornabene et al, 1983). This accumulation of fatty acids is a response to stress-inducing conditions and functions as a way to store energy. A similar accumulation also occurs during cell division and is linked to microalgal growth stages. Under these conditions, however, the microalgal culture is slow-growing and therefore it would not be viable to directly exploit this

capability to accumulate fatty acids under stress conditions. However, if these mechanisms and the interplay between different metabolic pathways could be completely understood, strains could be engineered to retain a high growth rate while accumulating high levels of fatty acids in the form of TAG under the aforementioned conditions, for example under cold stress or nitrogen deprivation. Specialised cultivation of transgenic lines could stimulate the metabolic changes and bolster biomass and valuable metabolites.

A more viable route would be to exploit and enhance the natural LC-PUFA biosynthetic machinery in omega-3-rich species. The commonly occurring pathways and genes already present in microalgae and diatoms, coupled with these organisms' relatively simple fatty acid profiles and higher growth rates compared to plants, make them attractive candidates for metabolic engineering for the enhancement of the production of EPA and DHA-rich oil. Apart from their higher growth rates, microalgae also possess several other advantages over plants as a source of LC-PUFAs; a smaller area is required to produce the same biomass as a traditional oilseed crop (Schenk et al, 2008) and there would be no competition for arable land (Greenwell et al, 2010) or fresh water (Hu et al, 2008).

The sequencing of algal genomes has paved the way for the identification of genes encoding desaturases and elongases and their functional characterisation using model yeast and higher plant systems (Domergue et al, 2002; Meyer et al, 2003; Meyer et al 2004; Pereira et al, 2004; Domergue et al, 2005; Tonon et al, 2005a; Zhou et al, 2007; Hoffman et al, 2008; Robert et al, 2009; Petrie et al, 2009; Iskandarov et al, 2009; Iskandarov et al, 2010; Petrie et al, 2010; Vaezi et al, 2013). Although these efforts were primarily aimed to identify suitable candidate genes for expression in transgenic plants, these discoveries furthered our understanding of PUFA biosynthesis in marine microalgae. Genome sequencing of microalga, such as the green alga *C. reinhardtii* (Shrager et al, 2003; Merchant et al, 2007) and the diatoms *Phaeodactylum tricornutum* (Bowler et al, 2008) and *Thalassiosira pseudonana* (Armbrust et al, 2004), also led to the development of molecular biology techniques and tools which would allow the genetic modification of certain microalgal species. Research on these model species, has shed light on microalgal and diatom biology. A common trait is their complexity, which has revealed that although species may be evolutionarily close, their behaviour and metabolic characteristics can vary dramatically. This has meant that although transformation techniques exist for some species (see section 1.3), there is little transferability between species and each requires its own set of promoters, markers and screening assays. This lack of ubiquity renders understanding and manipulating the mechanisms responsible for PUFA biosynthesis an extremely complex task.

The first steps in engineering microalgae for improved PUFA production came from the identification of genes involved in the PUFA biosynthetic pathways. This began primarily to identify candidate desaturases and elongases for heterologous expression in higher plants (discussed above). The acyl-CoA- dependent nature of some algal front-end desaturases involved in PUFA biosynthesis has been explained in detail above (see section 1.2.2.2.1). Some examples of unusual algal desaturases, from two model diatoms, worth mentioning are:

1. FAD6 and FAD2 from *P. tricornutum* (Domergue et al, 2003) – this diatom is rich in EPA but only contains trace levels of DHA. The genes and pathways involved in EPA biosynthesis in *P. tricornutum* have been characterised (Domergue et al, 2002). FAD6 and FAD2 were found to code for a plastidial and microsomal Δ12-desaturase respectively. The high specificity of FAD6 to the supplemental substrate 16:3Δ9 during characterisation experiments indicated that this enzyme is of plastidial origin. Interestingly, the chloroplastic lipids of *P. tricornutum* contain an ω3 hexadecatrienoic acid (16:3Δ7,10,13) isomer, ω6 hexadecatrienoic acid (16:3Δ6,9,12), and ALA (18:3Δ9,12,15), which are normally found in the membrane lipids of higher plant chloroplast membranes. The plastidial nature of FAD6 was confirmed when expressed in *P. tricornutum* with a fused EGFP (enhanced green fluorescent protein). FAD2 was found to be specific for oleic acid (18:1Δ9), similar to other microsomal desaturases, and suggests that it is involved in EPA biosynthesis in the ER.
2. TpDESN from *T. pseudonana* (Tonon et al, 2004) – this unusual front end desaturase has the typical features of a microsomal desaturase involved in LC-PUFA biosynthesis: three conserved histidine-rich motifs and a fused N-terminal cytochrome b5 domain. However, this enzyme displayed Δ11-desaturase activity, something that had previously only been observed in insects. Rather than being active on supplemented PUFA substrates, this desaturase was specifically active on saturated 16:0, producing 16:1Δ11.

These unusual behaviours demonstrate how functionality cannot always be correlated to the presence of typical features, motifs or homologous sequences to known and characterised genes. *T. pseudonana* shares 57% of *P. tricornutum* genes (Bowler et al, 2008), so their diversity is to be expected. What is surprising is these unusual traits which they share with insects and higher plants, respectively. The pennate diatom *P. tricornutum* has a higher number of species-specific gene families, which suggests it also has more specialised

functions, whereas the centric diatom *T. pseudonana* shares more features with eukaryotes (Bowler et al, 2008). The rapid rates of divergence found in diatoms (Sims et al, 2006; Kooistra et al, 2007; Bowler et al, 2008), is due to a number of reasons; the rates of gene loss and gain are very similar between diatom-specific and non-specific genes, and these diatom-specific genes evolve faster than the other genes in the genome (Bowler et al, 2008). Furthermore, the rate of substitution is the fastest reported (Kooistra and Medlin, 1996). The phylogenetics must be kept in mind when characterising and manipulating metabolic pathways in microalgae, and particularly in diatoms.

This last point is further underlined when taking into consideration microalgal elongases. A unique Δ9-elongase was isolated from the microalga *Isochrysis galbana* (Qi et al, 2002) which is only able to elongate Δ9-desaturated C18 PUFAs, LA (18:2n-6, Δ9,12) and ALA (18:3n-3, Δ9,12,15), converting them to ω6-eicosadienoic acid (20:2n-6, Δ11,14) and ω3-eicosatrienoic acid (20:3n-3, Δ11,14,17) respectively. This elongase could not be grouped into an elongase phylogram and it was unable to be concluded whether it formed a separate branch of its own (Meyer et al, 2004). The phylogram produced by Meyer et al also yielded another interesting observation; Δ5-elongases from *O. tauri* and *T. pseudonana* are not closely related to either each other or to Δ6-elongases from the same two algae, suggesting that ancient gene replications may have occurred. The evolution of C18-PUFA elongation to DHA seems to have arisen via two separate routes. One of the routes utilises two distinct and specific Δ6 and Δ5-elongases to carry out consecutive elongation reactions. This pathway requires a Δ4-desaturase to produce DHA since elongation halts at C22-substrates. The second route, commonly known as the Sprecher pathway (Voss et al, 1991) and found in mammals and fish, involves a single multifunctional elongase enzyme and results in the chain elongation of C18, C20 and C22 substrates in the microsomes. The final C24 elongation product is beta-oxidized in the peroxisomes to yield DHA. This pathway does not require a Δ4-desaturase for the biosynthesis of DHA.

When considering the transgenic production of DHA (whether in higher plant or algal hosts) the Δ4-desaturase-dependent pathway is a more ideal option, primarily because it does not yield C24-intermediates and unwanted by-products which would occur with the broad specificity of elongases in the mammalian Sprecher pathway, particularly in a plant background whose fatty acid profile is more complex than that of algae. Furthermore, transgenic products meant for plant or animal consumption are unlikely to be accepted if they are the source of mammalian genes due to the fear over gene transfer (Meyer et al,

2004). Thus, even when engineering algae for enhanced EPA and DHA production, suitable candidate genes are required from a number of algal species.

A number of strategies could be suitable for engineering microalgae to increase the oil content and/or manipulate the oil composition. The most obvious approach is to target lipid biosynthesis directly and use either overexpression or knockout/down techniques to modify the lipid content. This strategy also helps to clarify the role of specific genes and further our understanding of PUFA biosynthetic pathways. Work in this area is still in the early stages, with only a few examples in a limited number of species. This can be attributed to the lack of transferability of methodology between algal species and strains since they are so diverse. Other approaches include altering lipid catabolism and directly modifying lipid characteristics through the expression of acyl-ACP thioesterases to control the chain length in fatty acids. Acyl-ACP thioesterases hydrolyse the acyl moiety of the fatty acid from the acyl-ACP, releasing free fatty acids (Spencer et al, 1978). Acyl-ACP thioesterases specific for short chain fatty acids have been used for the engineering of biofuels in microalgae where shorter chain fatty acids are more desirable (Radakovits et al, 2011).

The first example in the area of direct targeting of lipid biosynthesis was the overexpression of an acetyl-CoA carboxylase (ACCase) from the diatom *Cyclotella cryptica* (Dunahay et al, 1996). Using chimeric plasmid vectors containing the neomycin phosphotransferase gene conferring resistance to aminoglycoside antibiotics, Dunahay et al used particle bombardment to transform both *C. cryptica* and *Navicula sapuvila*, another diatom. Despite increased ACCase activity, no increase in lipid production was observed (Sheehan et al 1998). The first successful attempt at engineering the omega-3 trait in transgenic algae came recently with the overexpression of heterologous genes in the diatom *Phaeodactylum tricornutum* (Hamilton et al, 2014). *P. tricornutum* can accumulate EPA to levels up to 35% of total fatty acid content, but only trace amounts of DHA. Two genes from the picoalga *Ostreococcus tauri* encoding a Δ6-desaturase and Δ5-elongase were biolistically transformed into *P. tricornutum*. The group found that there was a significant increase in DHA following expression of the Δ5-elongase, and this level rose further with the co-expression of the acyl-CoA-dependent Δ6-desaturase, to up to eight-fold higher amounts of DHA when compared to wild type strains (Hamilton et al, 2014). Importantly, no detrimental effects to *P. tricornutum* growth rates were observed, they were comparable to that of wild type strains. The next step in this research is to determine whether co-expression of the Δ5-elongase and Δ6-desaturase with a DGAT gene can increase incorporation of the DHA into TAG. Overexpression of DGAT2 in *P. tricornutum* has already been found to stimulate the

production of lipid bodies, with a 35% increase of neutral lipid and a significant increase of EPA (Niu et al, 2013).

The combined action of anabolic and catabolic pathways, and their balance, determines the overall net accumulation of a metabolic product (Bates et al, 2014). The targeted knock-down of lipid catabolism could potentially improve the accumulation of free fatty acids. *T. pseudonana* has been engineered using antisense and RNAi constructs to disrupt lipid catabolism by targeting an enzyme with lipase activity (Trentacoste et al, 2013). This resulted in an increase in the accumulation of TAG and total lipid yield without impacting growth rate, under light:dark conditions, continuous growth and nutrient replete vs. nutrient deficient conditions. The authors did not investigate the differences in TAG species between knock-down and wild type strains, but FAME profiling revealed that knock-down strains contained more 16:0 and 18:2 than wild type species. The mechanisms responsible for this skewing of the FAME profile are unclear and as yet it cannot be concluded if altering lipid catabolism can be targeted in a way to accumulate specific fatty acids in TAG. It is likely that a combination of strategies will the most feasible approach to improve LC-PUFA production and accumulation in TAG without inhibiting growth rates.

Although in its infancy, rational metabolic engineering of microalgae has a promising future for enhanced lipid accumulation, be that for biofuel production or valuable fatty acids. Progress in this area is already seeing exponential improvement and the successes of lipid engineering in higher plants gives an insight into what can yet be accomplished.

## 1.3 GENETIC TRANSFORMATION OF MICROALGAE

Microalgae are a phylogenetically heterogeneous group of functionally diverse photosynthetic eukaryotes. Advances in microalgal genomics have greatly contributed to the development of genetic engineering techniques in microalgae. The availability of rapid large-scale sequencing technology has been revolutionary in this field of research. Several algal nuclear, chloroplast and mitochondrial genomes have been sequenced, with many others in progress. Genomic data and expressed sequence tag (EST) libraries serve to greatly expand the microalgal molecular toolbox.

The first demonstration of transformation in a cyanobacterium was in *Synechococcus elongatus* (strain PCC 7942) (*Anacystis nidulans R2*) (Shestakov and Khyen, 1970). The first successful transformation of a microalga was of the chlorophyte (green alga) *C. reinhardtii*,

with the development of both nuclear and chloroplast transformation techniques (Boynton et al, 1988; Blowers et al, 1989; Kindle et al, 1989). *C. reinhardtii* has historically been the focus of efforts in this area and has become the main model species in molecular biology in eukaryotic algae. It is therefore one of the best described and most of the genetic engineering tools have been developed for *C. reinhardtii*. These tools are also specific to this organism and not transferable to other algal species. However, with the sequencing of more algal genomes, molecular biology tools are also being rapidly developed for other microalgae and diatoms.

### 1.3.1 Genome sequencing and transcription factors

Systems biology, encompassing various '-omics' approaches, and the advances in next-generation sequencing technology have greatly aided microalgal research. Algal genome research is required to form the basis of the development of metabolic engineering and genetic manipulation methods in microalgae. Annotation of the sequenced genomes will enable rational engineering approaches since gene function is known, thereby avoiding some of the shortfalls of a random engineering approach. Several microalgae nuclear genomes have been sequenced, including model organisms *C. reinhardtii* (Shrager et al, 2003; Merchant et al, 2007), *T. pseudonana* (Armbrust et al, 2004) and *P. tricornutum* (Bowler et al, 2008). Others include the single-cell green picoalga *Ostreococcus tauri* (Derelle et al, 2006), *Ostreococcus lucimarinus* (Palenik et al, 2007), as well as other completed nuclear, plastid and mitochondrial genomes, and several ongoing sequencing projects for each of these (Lluisma and Ragan, 1997; Crépineau et al, 2000; Nikaido et al, 2000; Shimko et al, 2001; Scala et al, 2002; Archibald et al, 2003; Bachvaroff et al, 2004; Henry et al, 2004; Matsuzaki et al, 2004; Wahlund et al, 2004; Weber et al, 2004; Hackett et al, 2005; Maheswari et al, 2005; Pruitt et al, 2005; O'Brien et al, 2007; Liolios et al, 2008; Worden et al, 2009; Radakovits et al, 2012; Vieler et al, 2012).

These sequencing projects not only serve to expand the molecular toolbox available for different species, but also to reveal the evolution and origin of diversity amongst these organisms. By comparing genomes of different strains in one species, the elements responsible for particular traits can be determined and phylogenetic explanations for intra-strain differences can be ascertained (Wang et al, 2014). This furthers our understanding of the underlying mechanisms responsible for various metabolic pathways and physiological

behaviour. This can then be extended to inter-species comparisons and enable the identification of species and strains which are most suited for a particular application.

In higher plants genes encoding transcription factors have been recognised as an important source of diversity (Carroll, 2000; Riechmann et al, 2000). Identification of trans-elements and transcription factor binding sites allows the detailed examination of how metabolic networks are regulated and presents another rational target for the manipulation and engineering of these networks to enhance productivity (Kellis et al, 2003; Babu et al, 2003; Stark et al, 2007; Hu et al, 2014). Trans-elements and transcription factor binding sites can be identified on a genome-wide scale using computational and experimental techniques. *In silico* methods are valuable when experimental techniques can be costly and time-consuming. Such experimental methods include electrophoretic mobility shift assays (Garner and Revzin, 1981), DNAse footprinting (Galas and Schmitz, 1978) and high-throughput techniques such as ChIP-Seq (Jothi et al, 2008); the first two are time intensive and the last is costly.

Genome-wide identification of transcription factors in microalgae using computational methods has been reported in a limited number of species, including a number of *Nannochloropsis* strains (Vieler et al, 2012; Hu et al, 2014), *Volvox cartier*, *Galdiera sulphuraria* and the model green alga *C. reinhardtii* (Pérez-Rodríguez et al, 2010; Zhang et al, 2011), together with a systematic prediction of cis-regulatory elements in *C. reinhardtii* (Ding and Hu, 2012). Despite such progress, this area of research is still in its infancy and there is little information regarding the relationship between microalgal transcription factors and their corresponding binding sites. Recently, however, an inroad has been made in this area. Hu and colleagues (2014) used a phylogenomic approach to create a genome-wide *in silico* map of transcription factors and their binding sites in three strains of *Nannochloropsis*. Theirs is the first computational prediction of a regulatory network linking transcription factors and their target genes in oleaginous microalgae (Hu et al, 2014). These results will allow more rational engineering of metabolic pathways in *Nannochloropsis* for enhanced target molecule production, such as lipids.

### 1.3.2 Techniques for the transformation of microalgae

In order to rationally engineer microalgae, be it to introduce a novel trait or to improve or change an existing one, transformation methods must be developed. Such techniques were first developed in chlorophyta (green algae), primarily in *C. reinhardtii* (Eichler-Stahlberg et

al, 2009). These techniques were improved and the methodology has been utilised in other algal groups including diatoms, dinoflagellates, euglenoids, rhodophyta (red algae) and phaeophyta (brown algae) (Table 1).

The cell membrane must be permeated temporarily for genetic transformation to occur by allowing exogenous DNA molecules to enter the cell. A successful transformation event will occur when the cell is viable and there is incorporation of the exogenous DNA into the microalgal nuclear or chloroplast genome. Most cells, however, do not survive the cell rupture process, and even those that do may recognise the exogenous DNA as foreign and degrade it.

The diversity of microalgal species means that not all transformation methods work for all species. Depending on the species being transformed, there are a number of physical barriers to overcome before exogenous DNA can be integrated into an algal genome. These may include a cell wall (silica in the case of *T. pseudonana*) and a number of additional internal membranes. Furthermore, once the transgene has been integrated there are a number of factors which may occur in different species that can have a negative impact on its expression and the production of recombinant proteins. These include regulatory mechanisms (Choquet et al, 1998; Wostrikoff et al 2004; Minai et al, 2006), codon dependency (Gustafsson et al, 2004; Heitzer et al, 2007), gene silencing (Heitzer et al, 2007), protein localistation (Bock, 2001), sensitivity to proteases (Surzycki et al 2009), enhancer elements (Lumbreras et al, 1998; Berthold et al, 2002; Eichler-Stahlberg et al, 2009), availability of transcription and translation factors (Inaba and Schnell, 2008; Jarvis, 2008) and genotypic modifications associated with transformation (Surzycki et al 2009). It is worth noting that codon optimisation of microalgae-destined heterologous transgenes increases translation rates and decreases susceptibility to silencing (Heitzer et al, 2007). Codon optimisation is necessary for high level expression (Mayfield et al, 2003; Mayfield and Schultz, 2004) since codon usage is the most conserved factor contributing to protein expression in prokaryotic genomes (Lithwick and Margarit, 2003), such as microalgal chloroplasts (Surzycki et al 2009). Moreover, expression can be further stabilised with the use of strong endogenous promoters and species-specific intron, 5’ and 3’ sequences (Eichler-Stahlberg et al, 2009). The more genomes that are sequenced, the more endogenous promoters will be available, which will circumvent one of the problems of codon usage bias associated with the current lack of available promoters (Schuhmann et al, 2012).

When nuclear transformation methods are employed transgenes are integrated randomly. This doesn't always matter when overexpressing transgenes and can be useful for random mutagenesis screens; it does, however, make targeting of specific genes more complicated. Homologous recombination in nuclear genomes was primarily successful in *C. reinhardtii* (Zorin et al, 2009), but has since been reported in a number of other microalgae (Kilian and Vick, 2011; Kilian et al, 2011; Weeks, 2011; Lozano et al, 2014). Other mechanisms for the targeted knock-down/out of endogenous genes include RNA silencing using RNAi and antisense RNA techniques (Casas-Mollano et al, 2008; De Riso et al, 2009; Liu and Benning 2013), and high-throughput artificial-micro RNA (armiRNA) techniques (Molnar et al, 2009; Zhao et al, 2009).

For some recombinant proteins their transgene expression and protein localisation is required in the chloroplast in order for them to function correctly. Chloroplast transformation is possible in *C. reinhardtii* through homologous recombination (Marín-Navarro et al, 2007). A similar method has recently been reported for the diatom *P. tricornutum* (Xie et al, 2014) as well as other homologous recombination techniques in the chloroplast (Oey et al, 2014). Plastid targeting sequences have also been used to localise a recombinant protein to the chloroplast following transformation (Apt et al, 2002; Gruber et al, 2007).

Transformation efficiency appears to be extremely species dependent (Radakovits et al, 2010), even if all the above factors have been taken into consideration. This is another contributory factor for lack of transferability of transformation techniques between species. Moreover, the limited availability of available markers presents another obstacle. The stable transformation of many species relies on the co-transformation of genes conferring antibiotic resistance (spectinomycin (Cerutti et al, 1997; Doetsch et al, 2001), nourseothricin (Zaslavskaia et al, 2000), G418 (Dunahay et al, 1995; Poulsen and Kröger, 2005; Zaslavskaia et al, 2000), bleomycin (Apt et al, 1996; Fischer et al, 1999; Fuhrmann et al, 1999; Lumbreras et al, 1998; Zaslavskaia et al, 2000), streptomycin (Doetsch et al, 2001), paromomycin (Jakobiak et al, 2004; Sizova et al, 2001) and chloramphenicol (Tang et al, 1995)), whereas auxotrophic markers are only available for a few species, for example *C. reinhardtii*. Furthermore, some antibiotics that are routinely used in plant transformations, such as hygromycin and kanamycin, are sensitive to NaCl concentration and therefore cannot be used with algal cultures that require sea water. Additionally, since algae are often naturally resistant to a wide range of antibiotic compounds, the number of antibiotics that work with

a specific strain will be much more limited, so this is neither the most suitable nor universal selection marker (Rehstam-Holm and Godhe, 2003). Other selective markers that have been used in microalgae include green fluorescent protein (GFP) (Fuhrmann et al, 1999; Zaslavskaia et al, 2000; Cheney et al, 2001; Ender et al, 2002; Franklin et al, 2002; Poulsen and Kröger, 2005), β-galactosidase (Gan et al, 2003; Jiang et al, 2003; Qin et al, 2003), β-glucuronidase (Kubler et al, 1994; Chow et al, 1999; El-Sheekh, 1999; Falciatore et al, 1999; Chen et al, 2001; Cheney et al, 2001) and luciferase (Jarvis and Brown, 1991; Falciatore et al, 1999; Fuhrmann et al, 2004). A chosen transformation method must be optimised not only for each species, but also for new strains. There are a number of established transformation methods for microalgae.

Particle bombardment and electroporation are the most frequently used transformation methods. Others include *Agroacterium tumefaciens*-mediated transformation and agitation in the presence of glass beads or silicon carbide whiskers. However, with the exception of an optimised glass bead-based technique in *Dunaliella salina* (Feng et al, 2009), these generally have lower transformation efficiencies than electroporation or particle bombardment.

| Species | Transformation method | Reference |
|---|---|---|
| *Chlamydomonas reinhardtii* | Agitation (glass beads) | Kindle, 1990 |
| *Patiria miniata* | Biolistic bombardment | Kurtzman and Cheney, 1991 |
| *Chlorella ellipsoidea* | Sonication | Jarvis and Brown, 1991 |
| *Chlamydomonas reinhardtii* | Agitation (silicon carbide whiskers) | Dunahay et al, 1993 |
| *Chlorella saccharophila* | Electroporation | Maruyama et al, 1994 |
| *Volvox carteri* | Biolistic bombardment | Schiedlmeier et al, 1994 |
| *Cyclotella cryptica* | Biolistic bombardment | Dunahay et al, 1995 |
| *Navicula saprophila* | Biolistic bombardment | Dunahay et al, 1995 |
| *Phaeodactylum tricornutum* | Biolistic bombardment | Apt et al, 1996 |
| *Ulva lactuca* | Electroporation | Huang et al, 1996 |
| *Chlorella sorkiniana* | Biolistic bombardment | Dawson et al, 1997 |
| *Amphidinium sp.* | Agitation (silicon carbide whiskers) | Michael and Miller, 1998 |
| *Symbiodinium microadriaticum* | Agitation (silicon carbide whiskers) | Michael and Miller, 1998 |
| *Chlamydomonas reinhardtii* | Electroporation | Shimogawara et al, 1998 |
| *Chlorella vulgaris* | Electroporation | Chow and Tung, 1999 |
| *Chlorella kessleri* | Biolistic bombardment | El-Sheekh, 1999 |
| *Chlorella vulgaris* | Agitation (glass beads) | Hawkins and Nakamura, 1999 |
| *Chlorella sorokiniana* | Agitation (glass beads) | Hawkins and Nakamura, 1999 |
| *Chlamydomonas reinhardtii* | Agitation (glass beads) | Fuhrmann et al, 1999 |
| *Undaria pinnatifida* | Biolistic bombardment | Qin et al, 1999 |
| *Phaeodactylum tricornutum* | Biolistic bombardment | Zaslavskaia et al, 2000 |
| *Chlorella ellipsoidea* | Electroporation | Chen et al, 2001 |
| *Euglena gracilis* | Biolistic bombardment | Doetsch et al, 2001 |
| *Porphyra yezoensis* | Agrobacterium-mediated | Cheney et al, 2001 |
| *Phaeodactylum tricornutum* | Biolistic bombardment | Apt et al, 2002 |
| *Chlamydomonas reinhardtii* | Biolistic bombardment | Franklin et al, 2002 |
| *Porphyridium sp.* | Biolistic bombardment | Lapidot et al, 2002 |
| *Haematococcus pluvialis* | Biolistic bombardment | Teng et al, 2002 |
| *Laminaria japonica* | Biolistic bombardment | Jiang et al, 2003 |
| *Gracilaria changii* | Biolistic bombardment | Gan et al, 2003 |
| *Chlamydomonas reinhardtii* | Agitation (glass beads) | Fuhrmann et al, 2004 |
| *Chlamydomonas reinhardtii* | Agrobacterium-mediated | Kumar et al, 2004 |
| *Cyanidioschyzon merolae* | Electroporation | Minoda et al, 2004 |

| | | |
|---|---|---|
| *Cylindrotheca fusiformis* | Biolistic bombardment | Poulsen and Kröger, 2005 |
| *Dunaliella salina* | Electroporation | Sun et al, 2005 |
| *Dunaliella salina* | Biolistic bombardment | Tan et al, 2005 |
| *Thalassiosira pseudonana* | Biolistic bombardment | Poulsen et al, 2006 |
| *Dunaliella viridis* | Electroporation | Sun et al, 2006 |
| *Phaeodactylum tricornutum* | Biolistic bombardment | Gruber et al, 2007 |
| *Nannochloropsis oculata* | Electroporation | Chen et al, 2008 |
| *Chlamydomonas reinhardtii* | Agitation (glass beads) | Molnar et al, 2009 |
| *Nannochloropsis sp. W2J3B* | Electroporation | Kilian et al, 2011 |
| *Nannochloropsis gaditana* | Electroporation | Radakovits et al, 2012 |
| *Chlamydomonas reinhardtii* | Biolistic bombardment | Oey et al, 2014 |

**Table 1:** Algal species with established transformation methods. This list is not exhaustive.

#### 1.3.2.1 Particle bombardment

This transformation method uses heavy metal particles, such as gold or tungsten, to introduce recombinant DNA into cells. The particles are coated in the exogenous DNA and delivered to the cells using a biolistic delivery system which accelerates the particles at the cells with pressurised gas. The acceleration of the DNA-coated particles allows the cell wall, if present, and cell membranes to be bypassed. This technique is particularly useful for transforming microalgae such as the diatom *T. pseudonana*, which has a rigid silica cell wall. It is also useful for chloroplast and mitochondrial transformations as it allows the particles to be forced through the various membranes. One of the drawbacks of this method is that the equipment and reagents required can be costly (Table 1).

#### 1.3.2.2 Electroporation

The electroporation method has been used for the transformation of cells for over twenty years (Neumann et al, 1982). High intensity electrical field pulses are applied to the cells to temporarily disrupt lipid bilayers and allow molecules to pass the cell membrane. Electroporation can be used on naturally or chemically cell wall-deficient strains. Optimised electroporation methods in microalgae can have high transformation rates, rates of up to 2 x $10^5$ transformants per microgram of DNA have been reported in *C. reinhardtii* (Shimogawara et al, 1998). Optimal efficiency is affected by several factors: concentration of exogenous DNA, temperature, osmolarity and the capacitance and voltage of the electric

current used. The optimal value for each of these factors varies between species (Maruyama et al, 1994; Chen et al, 2001; Minoda et al, 2004; Sun et al, 2005; Chen et al, 2008) (Table 1).

#### 1.3.2.3 Agitation methods

These methods are relatively simple and inexpensive, but can only be used with cell wall-deficient strains. Cells are agitated in the presence of glass beads (Kindle, 1990) or silicon carbide whiskers (Dunahay et al, 1993) with recombinant DNA and polyethylene glycol (PEG), the latter increases transformation efficiency. Cell membranes are perforated by the velocities and collisions of the beads and cells. Glass beads are preferred due to the lower transformation rates, higher costs and health risks associated with silicon carbide whiskers (Potvin and Zhang, 2010). Agitation with glass beads has been shown to be more efficient for the transformation of *Dunaliella salina* than particle bombardment (Feng et al, 2009).

#### 1.3.2.4 *Agrobacterium tumefaciens*-mediated transformation

*Agrobacterium tumefaciens*-mediated gene transfer is a well-established transformation method in plants. *A. tumefaciens* transfers T-DNA to the target cell which stimulates cell division. T-DNA and virulence factors (vir) are located on the *A. tumefaciens* tumour inducing plasmid (pTi). Any DNA that is located between the T-DNA flanking regions is processed and transferred by the vir system. *A. tumefaciens*-mediated transformation has been demonstrated in microalgae, but is not used as widely as the other methods listed above. *C. reinhardtii* was successfully transformed using this method, with the observation that *A. temefaciens*-mediated transformation resulted in a fifty-fold increase of viable transformants compared to the agitation with glass beads method (Kumar et al, 2004).

### 1.3.3 New developments and future prospects

Although stable transformation has only been achieved in a limited number of species, the field of microalgal genetic engineering has advanced significantly and continues to do so exponentially (Fuhrmann, 2002), particularly with the rising number of species and strains which are able to be transformed (Table 1). Moreover, the development of more sophisticated techniques and advanced genetic tools will expand the microalgal molecular toolbox even further. Such examples that have already been developed in higher plants and could prove important in microalgal engineering include genome-editing tools, site-specific recombinases, tuneable transcription factors and synthetic promoters (Liu et al, 2013). Other examples, which have recently been optimised in algae, include the use of zinc-finger

nucleases (Sizova et al, 2013), high throughput genotyping of algal mutants (Zhang et al, 2014) and TALEs and TALENs (Gao et al, 2014; Weyman et al, 2014). Increasing demands for the commercialisation of microalgal products is a driving factor in this area. Furthermore, with techniques developing and evolving, metabolic engineering of microalgae could prove to be an invaluable tool in the development of economically viable value added algal products.

## 1.4 THE DIATOM THALASSIOSIRA PSEUDONANA

### 1.4.1 Introduction to Diatoms

*Thalassiosira pseudonana* (Hasle and Heimdal, 1970) is the marine diatom of interest that will be focused on in this research project. Diatoms are unicellular, photosynthetic, eukaryotic algae, found in freshwater and oceanic environments. They are extremely important in global carbon cycling, carrying out approximately one fifth of photosynthesis globally, generating as much carbon in the sea as that of all terrestrial rainforests combined (Field et al, 1998). However, the organic carbon produced in the oceans is consumed more rapidly than that produced terrestrially. Diatoms are responsible for around 40% of primary aquatic production, thus serving as a vital base for energy-efficient aquatic food webs.

*T. pseudonana* is a subclass of Coscinodiscophyceae, belonging to the Bacillariophyta division. *T. pseudonana* is an experimental model for diatom physiology, visually recognisable by intricate silica nanostructures as part of its cell wall, or frustule. The intricate patterns that are found on the frustule of all diatoms are species-specific and are genetically controlled (Armbrust, 2009). It is important to keep in mind that diatoms require a silica source in order to synthesise their silicified frustule; in their natural environment this is derived from silicic acid – silicon which has dissolved in the sea water.

### 1.4.2 Diatom Physiology

Diatoms contain the same organelles as other eukaryotic algae. The hallmark of the diatom is its cell wall which is highly differentiated and heavily impregnated with silica. Si uptake and deposition involves less energy expenditure than the formation of equivalent organic cell walls (Raven 1983). The wall is multipartite, always consisting of two large intricately sculptured units called valves, together with several thinner linking structures called girdle elements of cincture. The wall components, often loosely called the frustule, fit together very

closely so that flux of material across the wall must take place mainly via pores or slits in the components themselves. The cytoplasm is totally protected, in spite of the multiplicity of cell wall components (as many as 50 in some diatoms) (Round et al, 2007). Besides silica the wall also contains organic material which forms a thin coating around the valves and girdle elements and often a discrete layer beneath them.

*T. pseudonana* is a centric diatom with radial symmetry, appearing circular from above, and rectangular when viewed from the side, with two valves (two unequal halves of the cell wall) which fit together, like two halves of a petri dish. A high degree of organisation is present in the cell wall structure, which is accompanied by great precision and order in the formation of the wall (Round et al, 2007). Each frustule has one valve formed just after the last cell division and an older valve, which may have existed for several or many cell cycles. The girdle elements also differ in age. One set of elements is associated with the older valve and one with the newer. Each cell wall then consists of two halves. The older valve together with the girdle elements associated with it, is called the epitheca, while the newer valve and its associated elements is the hypotheca. The two valves are termed the epivalve and the hypovalve and the two sets of girdle elements are the epicingulum and hypocingulum (collectively cincture or girdle). It is convenient to refer to the siliceous part of the cell wall collectively as the frustule.

The two valves (the larger epitheca and the smaller hypotheca) are held together by siliceous hoops, or girdle bands, and new bands are formed during cell growth (Hildebrand et al, 2009). The glass-like frustule has attributed diatoms with an interesting life-cycle. Diatom reproduction is primarily through vegetative mitotic divisions (Figure 5). However, physical and developmental constraints associated with the siliceous frustule mean that successive divisions result in just one daughter cell being the same size as the parent cell, the second is slightly smaller. This happens because each daughter cell inherits one maternal valve as its epitheca and synthesises its own hypotheca *de novo*. As a result, the cell which inherits the maternal hypotheca is smaller than the maternal cell and the other daughter cell.

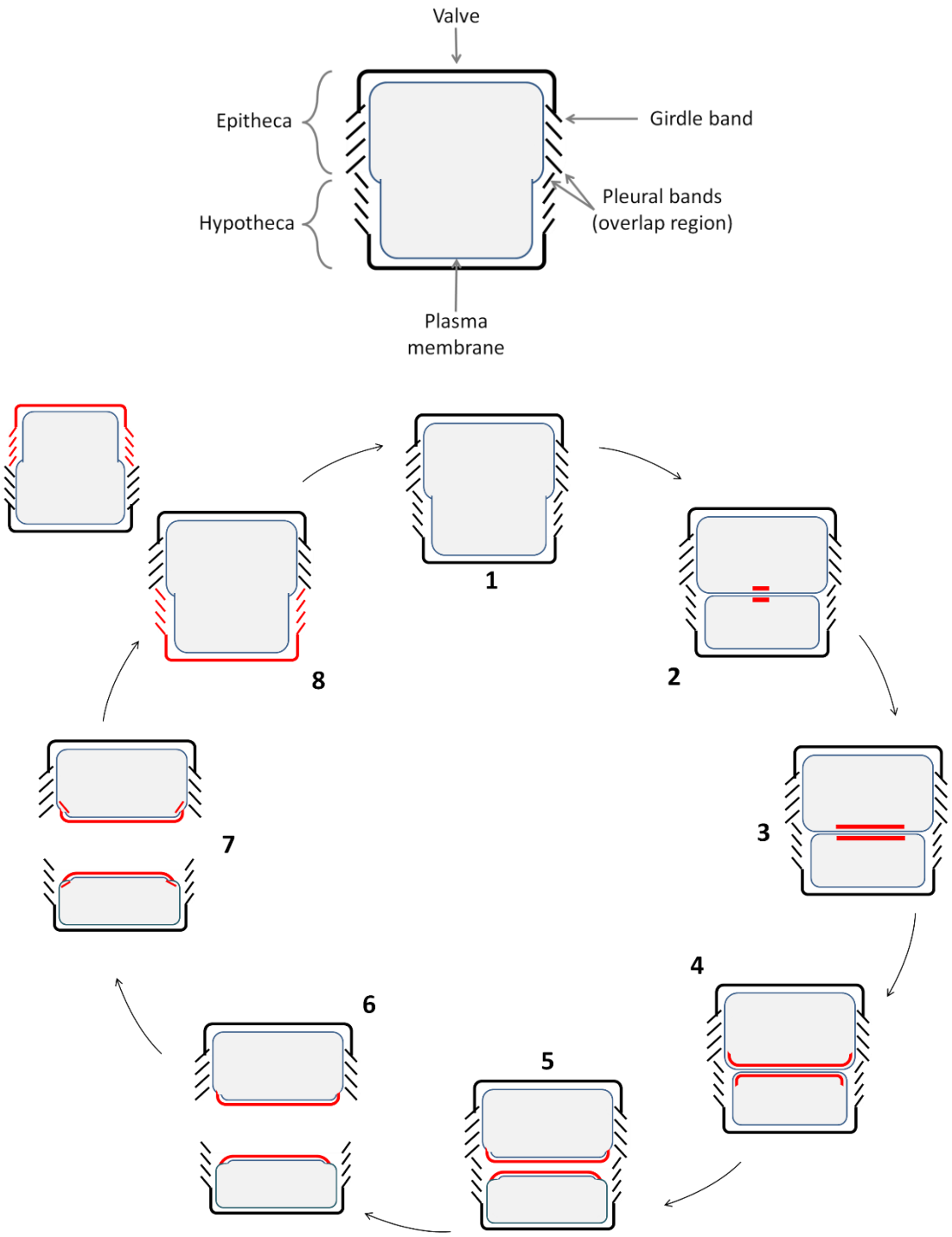



**Figure 5:** Schematic structure of *T. pseudonana* cell and vegetative cell cycle. The cross-section of cells shows the protoplast (shaded), plasma membrane (blue line) and silica cell wall (bold black line). The cell contains the maximum number of girdle bands just before cell division (1); new biosilica is formed (red) in each sibling cell immediately after cytokinesis (2); more silica is deposited (3), until a new fully developed valve is formed in each sibling cell (4); the newly formed valves are deposited in the furrow on the surface of each protoplast by exocytosis (5) and the sibling cells separate (6); expansion of the protoplast in interphase leads to the formation of new silica in the form of girdle bands (red dashes) which is exocytosed and added to the newly formed valve (hypovalve) (7); after the final girdle band (pleural band) of the hypovalve is synthesised, cell expansion stops and DNA replication is initiated (8). [Adapted from Kröger and Poulsen (2008)]

New parts of the cell wall are formed within the protoplasts and are then added to the wall by a form of exocytosis. This means that new valves and new girdle elements are necessarily smaller than the parts of the cell wall immediately outside them during their formation. Thus, because the girdle is usually parallel-sided and cylindrical, the hypovalve of a cell is usually smaller than the epivalve. The hypotheca always underlaps the edge of the epitheca, like the two halves of a petri dish. The valves, and to a lesser extent the girdle elements, are fairly rigid structures while surrounding the living cell, and so cell growth can only occur in one direction as the epitheca and hypotheca move apart. As this occurs, more elements are added to the hypocingulum, to accommodate the further expansion of the cell. The hypocingulum is often incomplete until just before cell division, when the last girdle elements are arranged. When the cell divides, the hypocingulum of the parent cell becomes the epicingulum of one daughter cell, and the parental epicingulum becomes the epicingulum of the other daughter cell. Once incorporated in an epitheca, a cingulum will never be added to. It may conceivably lose elements (although this is unusual) but new ones will not be formed to replace them (Round et al, 2007). This is because the girdle elements themselves are formed in sequence, from the valve outward (i.e. towards the middle of the cell). Thus, since the edge of the epicingulum is underlain by the hypocingulum, no girdle element can be formed, exocytosed and then transported between epicingulum and hypocingulum to take up a position at the edge of the epicingulum (Figure 5). The production of new frustule elements within the confines of the parental cell wall usually leads to a decline in mean cell size, one of the best known features of diatom biology.

There is therefore a huge variation in cell size within a population. Cell size must eventually be restored; this occurs via an auxospore – a special cell which develops and expands in a highly controlled manner before producing a new frustule. Auxospore formation is usually associated with sexual reproduction (Figure 6). In centric diatoms such as *T. pseudonana*, the sexual reproduction step to prevent diminishing cell size is triggered by numerous environmental cues, such as light conditions, nitrogen deprivation and cell density (Amato, 2010). Cells respond to these triggers by becoming either a sperm or egg cell, since centric diatoms are monoecious. The egg cell remains encased in the cell wall, whereas the sperm cell sheds its frustule. It has been postulated that a pheromone-like compound allows the egg cell to attract the sperm (Chepurnov et al, 2004); without such signals, the sperm would be unable to distinguish between egg cells and vegetative cells and also other egg and vegetative cells of other centric species. Furthermore, there would be the risk that the sperm cells would not find the egg in the ocean environment and both gametes would die. A series

of signalling events allow the sperm to access the egg by passing through the frustule. The gametes fuse to form a zygote, which swells to form a specialised cell – an auxospore. When the auxospore sheds its frustule it becomes a postauxospore which generates a new, much larger, frustule, thus restoring cell size. Asexual reproduction is rapidly resumed until iterative mitotic events again lead to a sexual reproduction event to overcome miniaturisation. Sexual reproduction introduces genetic diversity, boosts heterozygosity and also allows selectively advantageous genotypes to be transferred more rapidly throughout the population (Mann, 1993; Armbrust, 1999; Armbrust and Galindo, 2001; Round et al, 2007).

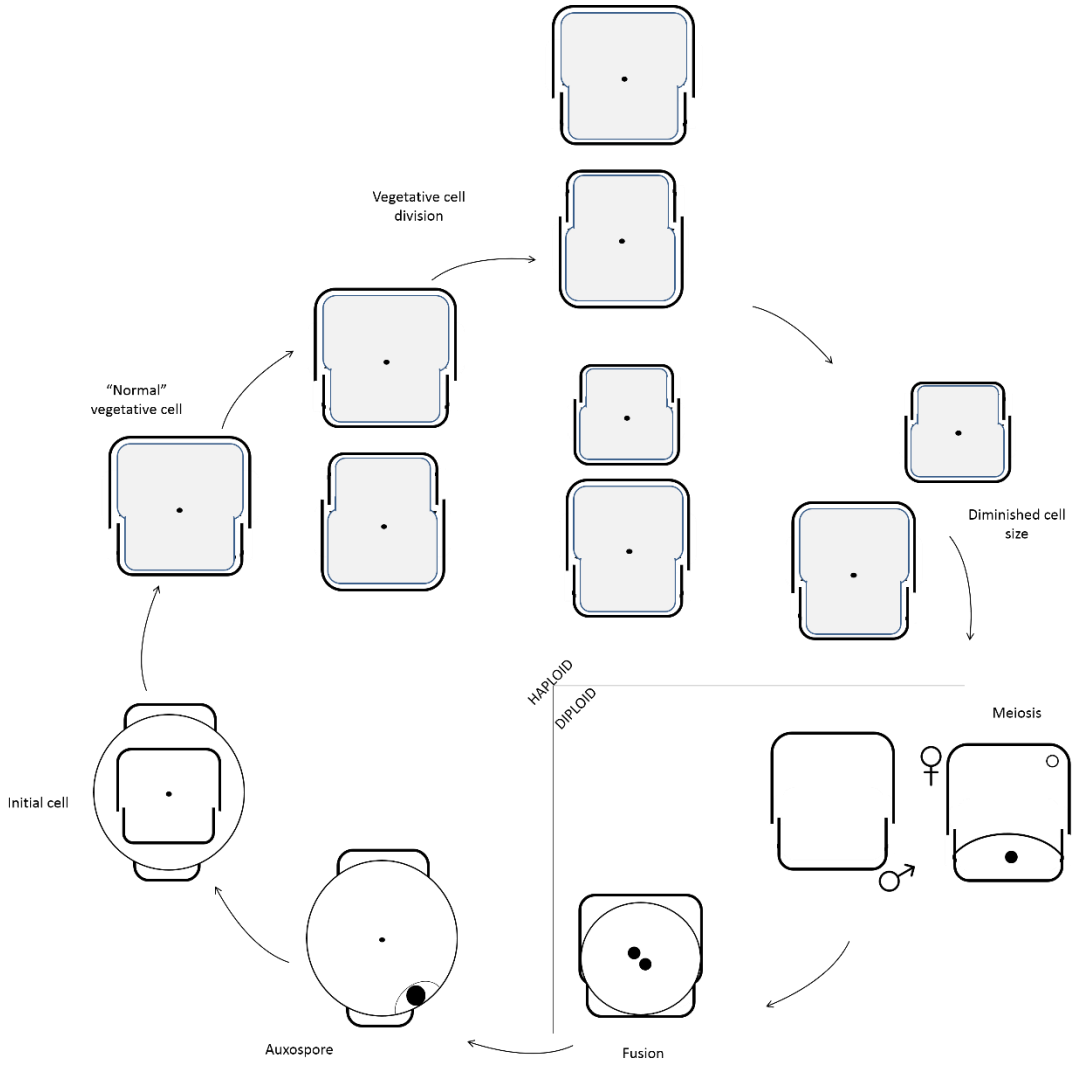



**Figure 6:** Schematic structure of *T. pseudonana* sexual reproduction cycle (oogamy). Normal vegetative cell division proceeds until cell size diminishes, leading to sexual reproduction via the formation of an auxospore in order to restore cell size. The sperm and egg gametes fuse to form a zygote, which swells to produce an auxospore. Once the auxospore sheds its frutule it becomes a post-auxospore and generates a new much larger frustule, thus restoring normal cell size and vegetatitve cell division ensues. [Adapted from Hasle et al (1996)]

Except during sexual reproduction, the diatom protoplast is completely contained within the silicified cell wall. The most conspicuous components of the protoplast are the plastids (chromatophores), which are usually brown because the carotenoid pigments (principally β-carotene, diatoxanthin, diadinoxanthin and fucoxanthin (Goodwin, 1974)) mask the colour of the chlorophyll. If cells are damaged or treated with acid, they turn green as the carotenoids are destroyed and the chlorophyll is unmasked (Round et al, 2007).

Plastids, mitochondria, dictyosomes and other organelles are usually packed into a fairly thin peripheral layer of cytoplasm sandwiched between the cell wall and one or two large central vacuoles. The nucleus is quite easy to see and either is suspended in the centre of the cell by a bridge or strands of protoplasm, or lies to one side of the cell, by the girdle or near one valve. It always changes position prior to cell division, unless it already lies alongside the girdle during interphase. Lipid bodies, polysaccharide reserves and other inclusions are often visible in the cytoplasm.

### 1.4.3 Evolution of Diatoms

Diatoms have significant importance from an evolutionary perspective due to their complex evolutionary history. Genome sequencing has further demonstrated the intriguing diversity of diatoms. The whole genomes of *T. pseudonana* and *P. tricornutum* have been sequenced (34 megabases and 27 megabases respectively) (Armbrust et al, 2004; Bowler et al, 2008). A comparison of their genomes revealed that, although they diverged from each other around 90 million years ago, their genomes show similar diversity to that between fish and mammals, which diverged 550 million years ago (Bowler et al, 2008). Furthermore, the presence of unique metabolic pathways and genetic combinations highlights how the separate evolutionary lineage giving rise to diatoms has distinguished them from land plants and other terrestrial photosynthetic organisms.

Different endosymbiotic events gave rise to diatoms and plants. Following primary endosymbiosis 1.5 billion years ago, a secondary endosymbiotic event occurred around 500 million years later (Figure 7). Algal nuclear and plastid genomes were transferred to the host heterotroph nucleus, resulting in the inclusion of algal genes in the diatom nuclear genome, the majority of which encode plastid components.

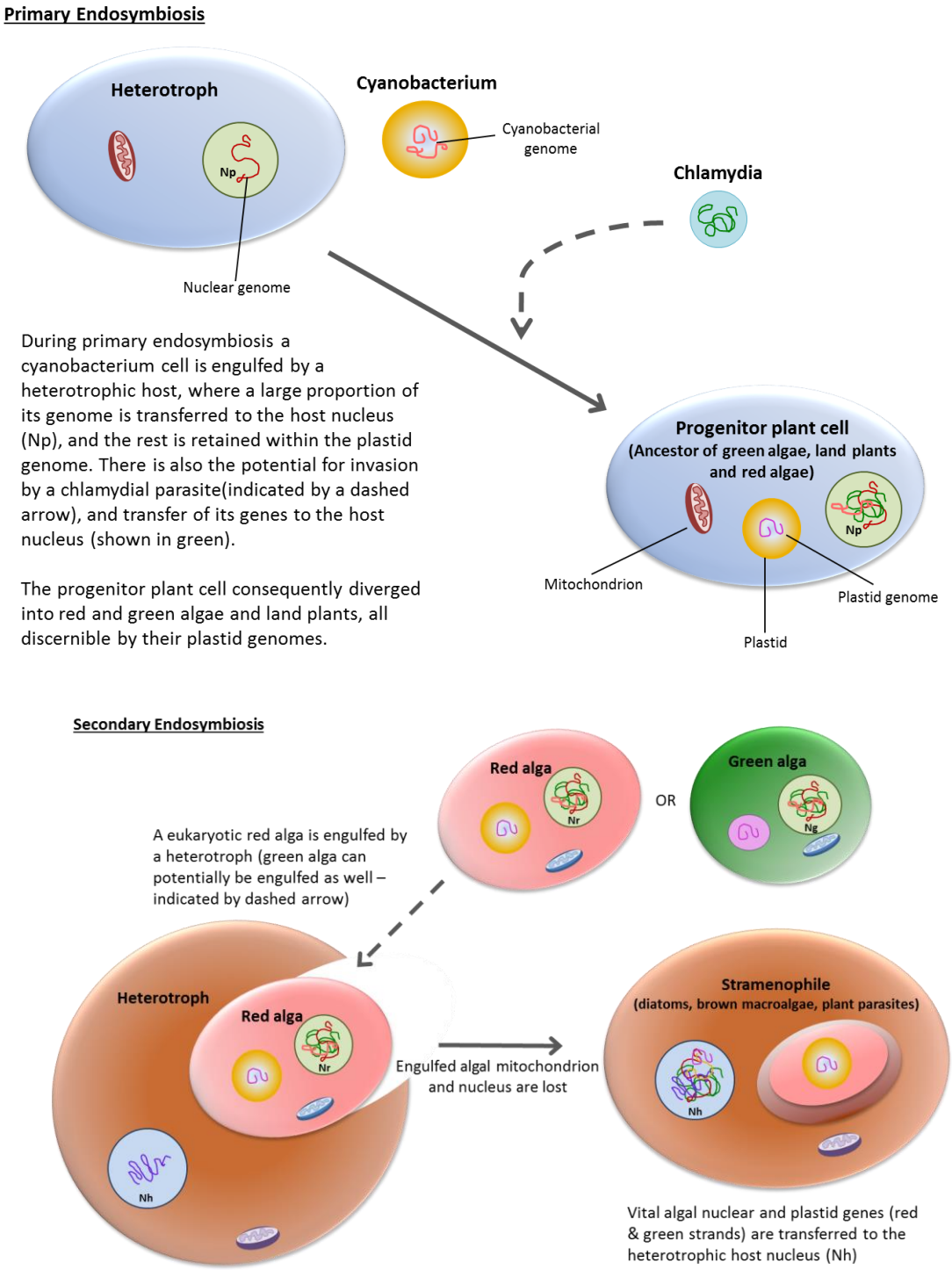



**Figure 7:** An overview of the evolution of diatoms following primary and secondary endosymbiotic events. The distinct lineage from other photosynthetic organisms and the origin of diatom plastids are shown. After secondary endosymbiosis, the algal nucleus and mitochondrion are lost. Further genes can be gained and lost from bacteria (not shown). [Adapted from Armbrust (2009)]

Diatoms have a combination of plant and animal metabolic characteristics – an animal-like ability to generate chemical energy from fat breakdown and a plant-like ability to generate metabolic intermediates through this breakdown, most likely to allow them to survive lengthy periods of darkness. Complementary characteristics have been compiled throughout their evolution, resulting in an organism which is neither plant nor animal; they have both the plant-like ability to photosynthesise, as well as an animal-like complete urea cycle. These attributes make diatoms particularly appealing for further research. They are also attractive candidates for metabolic engineering projects due to the vast range of valuable biological products that they produce, such as isoprenoids, fatty acids, triglycerides and antioxidants (Figure 8); these can be utilised in pharmaceutical, nutraceutical and biofuel applications, amongst others.

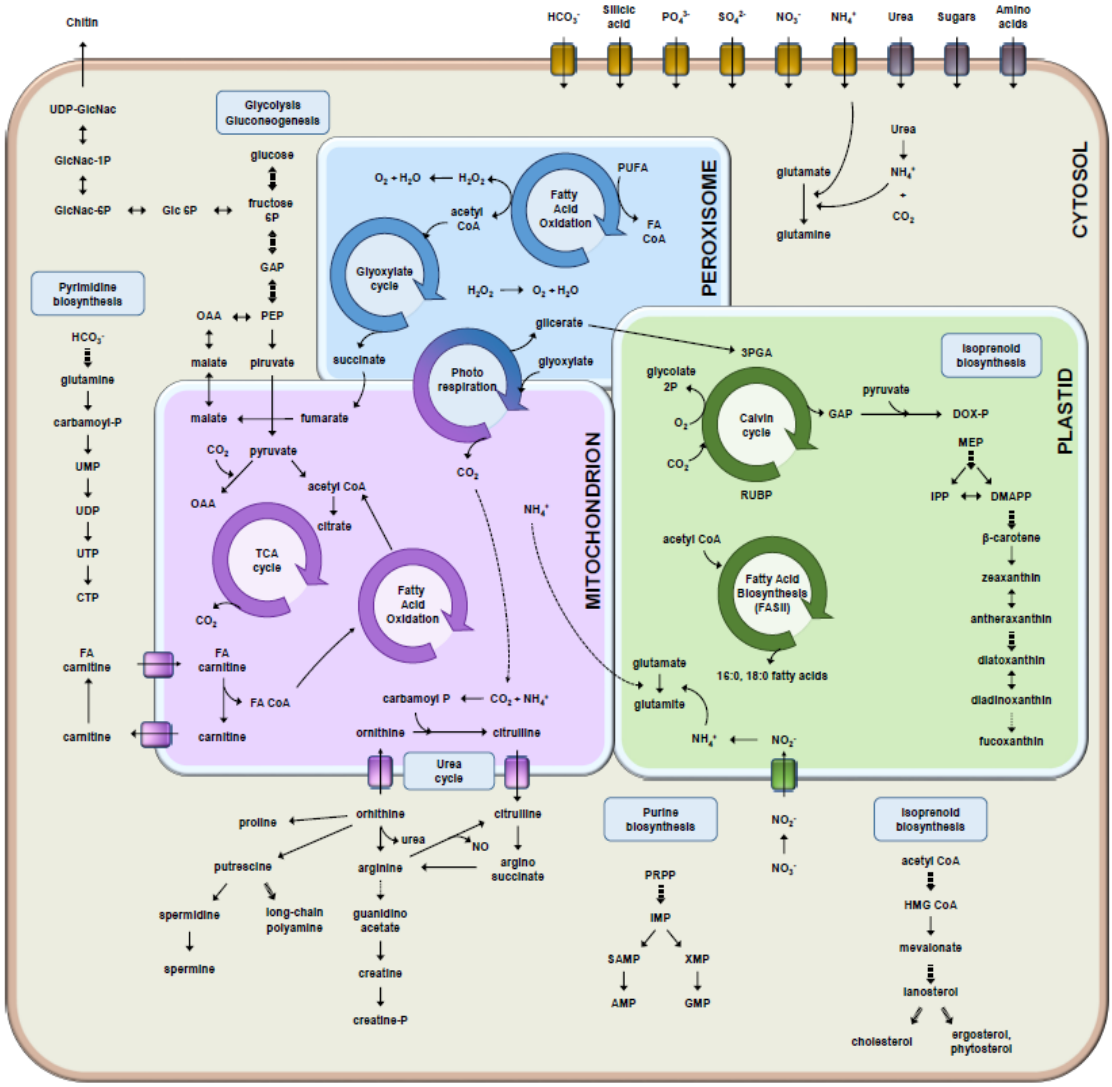



**Figure 8:** Overview of the metabolic pathways present in *T. pseudonana*. [Adapted from Armbrust et al (2004)]

Photosynthesis, biosynthesis of fatty acids, isoprenoids and amino acids occurs in membrane-bound plastids in *T. pseudonana* cells. The plastids appear as small disks in the cell, which fluoresce red when illuminated under blue light due to chlorophyll autofluorescence.

### 1.4.4 Rational for Selecting *T. pseudonana* as the Target for Omega-3 PUFA Enhancement

*T. pseudonana* is the main focus of this research for a number of reasons. Its nuclear, plastid and mitochondrial genomes have been fully sequenced (Armbrust et al, 2004), and some genes involved in PUFA biosynthesis have already been identified (Tonon et al, 2002; Armbrust et al, 2004; Meyer et al, 2004; Tonon et al, 2004; Tonon et al, 2005a; Tonon et al, 2005b). Furthermore, there is already some endogenous PUFA biosynthetic activity; polyunsaturated fatty acids comprise at least 25% of the fatty acids produced by *T. pseudonana*, including the health beneficial EPA and DHA (Volkman et al, 1989), the fatty acids of interest in this project. Moreover, transformation protocols and vectors already exist for this organism (Poulsen et al, 2006), which will be utilised for this project.

## 1.5 PROJECT RATIONAL AND RESEARCH OBJECTIVES

As mentioned above, marine microbes, particularly diatoms, are the primary producers of omega-3 fatty acids, so are attractive targets to study and engineer to produce these essential PUFAs for human consumption or as an animal feedstock. Work is already being undertaken to engineer higher plants to produce and accumulate omega-3 fatty acids (discussed in detail above). However, since marine microbes already produce and accumulate omega-3 fatty acids (as TAG), they are therefore a more attractive option for the development of an alternative large-scale sustainable source of omega-3s.

The omega-3 biosynthetic pathways are already present in *T. pseudonana* and thus pose as an attractive target to optimise for enhanced production, rather than engineering entire or partial production and accumulation pathways in organisms which do not already have these capabilities. Not only does this reduce the metabolic load on the organism, since it's not drastically altering its biosynthetic pathways, but it can also lead to better understanding of the organism's native metabalome. Furthermore, since microalgae are capable of surviving in seawater, future systems for large scale culturing of these organisms for omega-3 fatty

acid production would not drain freshwater reserves, which are required for human consumption and food production. Studies throughout this project will not only aim to engineer desirable activity in marine diatoms, but also to further understand algal molecular biology in order to facilitate research and application-driven projects on these organisms.

The development of a large-scale sustainable omega-3 PUFA production system is also incentivised from an economical standpoint. Omega-3 fatty acids are used in many industries, with a growing global market for omega-3 ingredients – market size in 2012 was €1.9 billion and is expected to reach €5.6 billion by 2023 (Frost & Sullivan, 2014). This market is a key example of a scientifically research driven global market; omega-3 fatty acids are one of the most extensively researched ingredients in the food and beverage industry.

The main objectives of this doctoral research project are threefold:

4) **To identify and functionally characterise algal genes involved in omega-3 biosynthesis.** This serves multiple purposes: firstly to provide genes that can be heterologously expressed in other organisms/other algal species; secondly, to expand the microalgal knowledge bank by providing gene/protein/behavioural information on a variety of functions from different sources; and finally, to further the understanding of the processes that govern omega-3 biosynthesis in microalgal species.
5) **To metabolically engineer *T. pseudonana* to enhance omega-3 production and/or accumulation.** In the first instance this will be attempted by the overexpression of heterologous genes. This study will also help to elucidate further information about lipid metabolic processes in *T. pseudonana*, with the aim of contributing further insight into lipid metabolism in microalgae.
6) **To silence/impair endogenous genes involved in omega-3 biosynthesis in *T. pseudonana*.** This study will be carried out with the aim of determining whether silencing of targeted genes has a positive or negative effect on omega-3 production/accumulation. Also to further understand the native omega-3 biosynthetic processes in *T. pseudonana*, again providing valuable insights into lipid metabolism in this diatom.

The following chapters detail the results obtained over the past four years, which are discussed in line with the objectives outlined above. Finally, an overview is provided summarising these results, with an integrative discussion including future research perspectives.

# Chapter 2

# Identification and Characterisation of Genes Involved In Omega-3 Biosynthesis

*Part of this chapter has been published as a paper entitled 'Identification and functional characterisation of genes encoding enzyme activities involved in omega-3 polyunsaturated fatty acid biosynthesis from unicellular microalgae' in the journal Marine Drugs (see Appendix i). Johnathan A. Napier and Olga Sayanova are co-authors of the paper. Alterations have been made to fit with this thesis and additional results and discussion have been included.*

## 2.1 ABSTRACT

In order to identify novel genes encoding enzymes involved in the biosynthesis of nutritionally important omega-3 long chain polyunsaturated fatty acids, a database search was carried out on the genomes of the unicellular photoautotrophic green alga *Ostreococcus* sp. RCC809 and cold-water diatom *Fragilariopsis cylindrus*. The search led to the identification of two putative "front-end" desaturases (Δ6 and Δ4) from *Ostreococcus* RCC809, a Δ5-elongase from *Ostreococcus* RCC809 and one Δ6-elongase from *F. cylindrus*. Heterologous expression of putative open reading frames (ORFs) in yeast revealed that the encoded enzyme activities efficiently convert their respective substrates: 54.1% conversion of α-linolenic acid for Δ6-desaturase, 15.1% conversion of 22:5n-3 for Δ4-desaturase, 51.6% conversion of ARA and 62.1% conversion of EPA for Δ5-elongase and 38.1% conversion of γ-linolenic acid for Δ6-elongase. The Δ6-desaturase from *Ostreococcus* RCC809 displays a very strong substrate preference resulting in the predominant synthesis of stearidonic acid (C18:4Δ6,9,12,15). These data confirm the functional characterisation of omega-3 long chain polyunsaturated fatty acid biosynthetic genes from these two species which have until now not been investigated for such activities. The identification of these new genes will also serve to expand the repertoire of activities available for metabolically engineering the omega-3 trait in heterologous hosts as well as providing better insights into the synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in marine microalgae.

## 2.2 INTRODUCTION

It is now well accepted that omega-3 long chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ4,7,10,13,16,19) are vital for human health and nutrition and play a crucial role in preventing cardiovascular diseases and associated precursor conditions such as metabolic syndrome and obesity (see Chapter 1 : Introduction, Section 1.2). However, as discussed in Chapter 1, current sources of these fatty acids have major drawbacks and there is an urgent need for an alternative cost-effective solution for the large-scale production of LC-PUFAs.

In recent years, the feasibility of using higher plants for the production of omega-3 LC-PUFAs has been explored and considerable progress has been made in effective production of EPA and DHA in oilseeds (Haslam et al, 2013; Ruiz-Lopez et al, 2013; Petrie et al, 2012). A variety of strategies have been used to introduce (via genetic engineering) the omega-3 LC-PUFA metabolic pathways in oil crops, mainly by expressing desaturase and elongase genes involved in different biosynthetic routes for EPA and DHA accumulation (Venegas-Calerón et al, 2010). Marine algae are the primary producers of omega-3 LC-PUFAs and therefore represent the logical source for the identification of genes encoding the enzymes required for the synthesis of EPA and DHA. Identification of these genes will also be advantageous for engineering marine microalgae.

Most omega-3 LC-PUFA-synthesising marine organisms utilise the so-called Δ6-desaturase "conventional" aerobic pathway which relies on a consecutive series of altering desaturation and elongation steps to convert α-linolenic acid (ALA; 18:3Δ9,12,15) to EPA and DHA (Figure 9). The first step in this pathway is the Δ6-desaturation of both linoleic acid (LA; 18:2Δ9,12) and ALA, resulting in the synthesis of γ-linolenic acid (GLA; 18:3Δ6,9,12) and stearidonic acid (SDA; 18:4Δ6,9,12,15), respectively. This step is followed by a Δ6-specific C2 elongation, yielding di-homo γ-linolenic acid (DGLA; 20:3Δ8,11,14) and eicosatetraenoic acid (ETA; 20:4Δ8,11,14,17). Finally, these LC-PUFAs are desaturated by a Δ5-desaturase to generate arachidonic acid (ARA; 20:4Δ5,8,11,14) and EPA, respectively. In DHA-accumulating microorganisms, the pathway involves C2 elongation of EPA to docosapentaenoic acid (DPA; 22:5 Δ7,10,13,16,19) by a specific Δ5-elongase which is then desaturated by a Δ4-specific desaturase to yield DHA. Although most enzymes involved in this pathway show limited discrimination between n-3 and n-6 acyl-substrates, the predominant presence of n-3 LC-PUFAs in most marine microorganisms indicates the likely presence of ω-3-desaturases which convert omega-6 to omega-3. However, some examples of Δ6-desaturases cloned from

microalgae show a preference for ω-3 substrates (also reported for some higher plant Δ6-desaturases from *Primula* sp and *Echium* (Sayanova et al, 2003; Sayanova et al, 2006; García-Maroto et al, 2006)). Microalgal enzymes with ω-3 substrate preference have also previously been described (*Mantoniella squamata* (Hoffmann et al, 2008), *Micromonas pusilla* (Petrie et al, 2010a) and *Ostreococcus lucimarinus* (Petrie et al, 2010b).

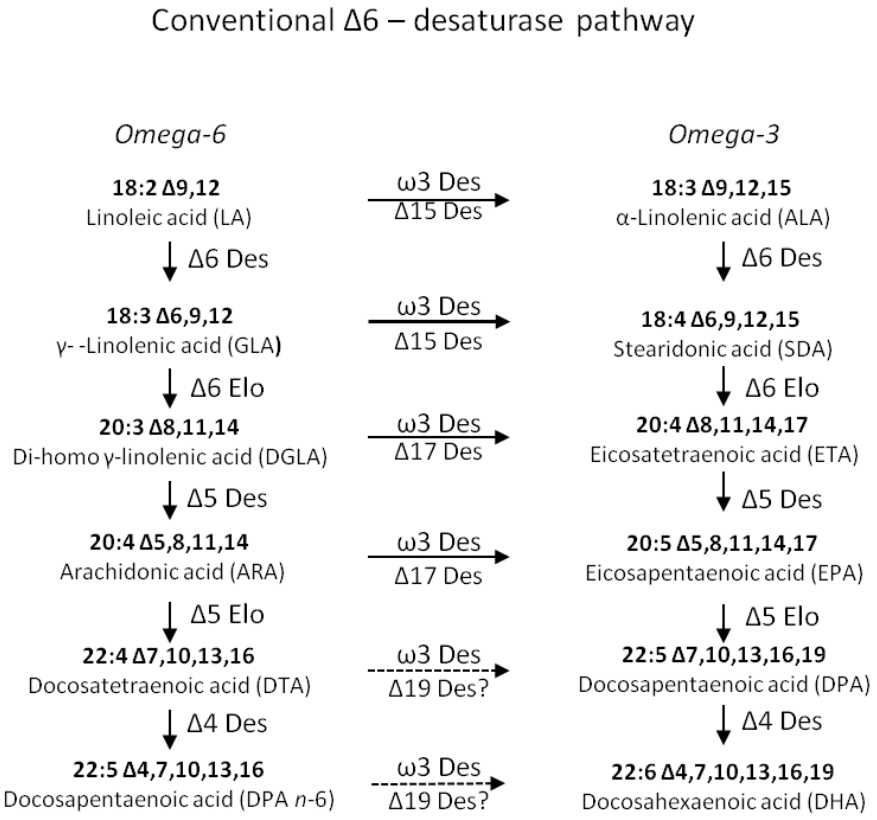



**Figure 9:** Conventional pathway for the biosynthesis of long chain polyunsaturated fatty acids (LC-PUFAs) in microalgae.

The fatty acid profiles of two marine microalgae were examined, namely the unicellular photoautotrophic green alga *Ostreococcus* sp. RCC809 and the cold-water diatom *Fragilariopsis cylindrus*, both of which have previously only been subjected to limited investigation as to the nature of their synthesis and accumulation of omega-3 LC-PUFAs. On-going genomic sequencing projects for these two organisms were used to identify and functionally characterise four examples of genes involved in the biosynthesis of EPA and DHA. Interestingly, one particular enzyme (Δ6-desaturase from *Ostreococcus* RCC809) showed a strong preference for ω-3 substrates versus ω-6.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Identification and Functional Characterisation of *Ostreococcus* RCC809 Genes for "Front-End" Desaturases

#### 2.3.1.1 Fatty Acid Composition of *Ostreococcus* RCC809

*Ostreococcus* RCC809 is the smallest known free-leaving marine picophytoeukaryote belonging to the "low-light" adapted ecotype of Prasinophyceae (Demir-Hilton et al, 2011). It is closely related to the two "high-light" adapted ecotypes of the genus *Ostreococcus*, *O. tauri* and *O. lucimarinus*, that have the ability to synthesise EPA and DHA via a series of alternating desaturation and elongation steps (Meyer et al, 2004; Domergue et al, 2005; Petrie et al, 2010b; Ahman et al, 2011; Demir-Hilton et al, 2011). To explore the LC-PUFA pathway operating in *Ostreococcus* RCC809, GC-FID and GC-MS were used to analyse the fatty acid methyl esters (FAMEs) of total lipids from *Ostreococcus* RCC809 cultures growing at stationary phase. This analysis revealed the presence of several fatty acids belonging to the n-3 PUFA pathway (Figure 10). The most abundant fatty acid was 16:0 (20.1% of total fatty acids, TFA) followed by SDA (19.1% of TFA) and 16:1 (18.1% of TFA) (Table 2). Compared to *O. tauri*, which contains on average 12% of DHA (Demir-Hilton et al, 2011), the amount of this fatty acid in *Ostreococcus* RCC809 is rather low (1.83%); similarly, the levels of EPA are very low. The fatty acid content of *Ostreococcus* RCC809 is comparable to what has been found for *O. lucimarinus*, containing high levels of SDA (15%) and small amounts of EPA and DHA (Wagner et al, 2010). Thus, the predominant omega-3 PUFA in *Ostreococcus* RCC809 is the Δ6-desaturated C18 fatty acid SDA, as opposed to either EPA or DHA.

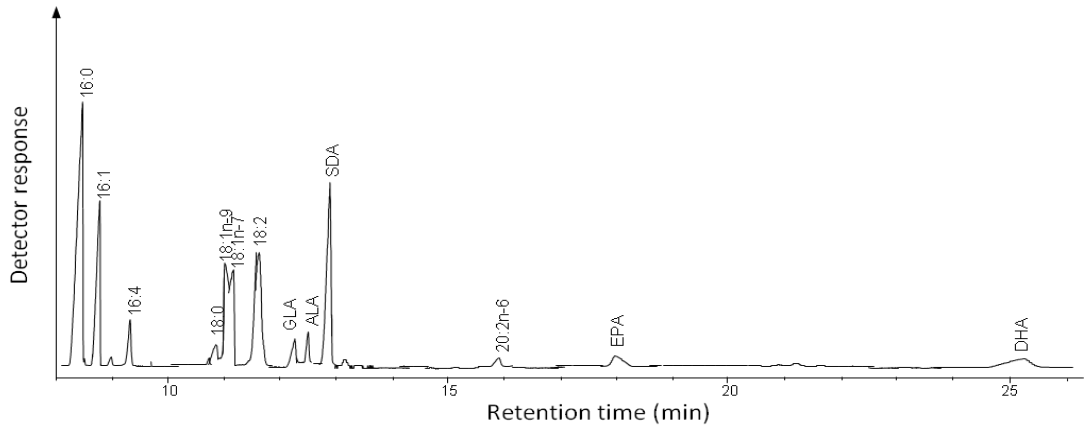



**Figure 10:** Total fatty acid methyl esters of *Ostreococcus* RCC809. Fatty acid methyl esters (FAMEs) were prepared and analysed by gas chromatography coupled with a flame ionisation detector (GC-FID), with peaks being identified by co-migration against known standards. The identity of major peaks is shown.

| | Composition (Molar %) ± SD | |
|---|---|---|
| **Fatty Acid** | ***O.* RCC809** | ***F. cylindrus*** |
| **16:0** | 20.12 ± 0.6 | 12.1 ± 0.2 |
| **16:1n-7** | 18.1 ± 0.3 | 24.5 ± 0.1 |
| **16:4n-3** | 5.08 ± 0.4 | ND |
| **18:0** | 1.24 ± 0.5 | 2.4 ± 0.3 |
| **18:1n-9** | 8.17 ± 0.2 | ND |
| **18:1n-7** | 8.03 ± 0.1 | ND |
| **LA** | 9.12 ± 0.2 | 0.7 ± 0.3 |
| **GLA** | 3.29 ± 0.3 | 1 ± 0.4 |
| **ALA** | 3.98 ± 0.4 | 0.8 ± 0.3 |
| **SDA** | 19.13 ± 0.6 | 5.7 ± 0.5 |
| **20:2n-6** | 0.65 ± 0.4 | 1 ± 0.2 |
| **20:4n-3** | ND | 7.4 ± 0.1 |
| **EPA** | 1.26 ± 0.4 | 31.4 ± 0.3 |
| **DHA** | 1.83 ± 0.5 | 2.5 ± 0.1 |
| **24:0** | ND | 1.4 ± 0.2 |

**Table 2:** Fatty acid composition of *Ostreococcus* RCC809 (*O.* RCC809) and *F. cylindrus* cultures in stationary phase. Values are the average of three independent experiments (± standard deviation). ND—not detected.

#### 2.3.1.2 Identification and Functional Characterisation in Yeast of a Putative Δ6-Desaturase from *Ostreococcus* RCC809

The genome of the green alga *Ostreococcus* RCC809 has been sequenced by US DOE Joint Genome Institute (JGI) and the predicted gene models are available for inspection and query (Joint Genome Institute *Ostreococcus* sp. RCC809 2.0 genome project). The predicted gene models were queried via BLAST using previously characterised N-terminal cytochrome b5-fusion desaturases as query sequences. This analysis revealed the presence of several genes coding for putative omega-3 LC-PUFA desaturases. The top-scoring predicted open reading frames (ORFs) are listed in Table 3. Interestingly, no obvious Δ5-desaturase was identified by the search, as might be expected for an organism that synthesises EPA (Figure 10, Table 2). The most obvious explanation for this absence is that the sequence of the *Ostreococcus* RCC809 genome is not yet complete and/or the genomic structure of the Δ5-desaturase is such that it has evaded the gene-prediction algorithms used to identify ORFs. The enzymatic activities of the two identified candidate desaturases (Table 3) were investigated by

heterologous expression in *Saccharomyces cerevisiae*, and the deduced ORFs were used as templates to chemically synthesise codon-optimised nucleotide sequences for expression in diatoms (based on the subsequent requirement to express the algal sequences in transgenic *T. pseudonana*, and the observation that such diatom codon usage was readily accepted by *S. cerevisiae*). These synthetic coding sequences were cloned as *KpnI-SacI* fragments behind the galactose–inducible GAL1 promoter of the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA) and expressed in yeast in the presence of potential fatty acid substrates as previously described (Sayanova et al, 2003). Total fatty acid methyl esters (FAMEs) from transgenic yeast were analysed by GC-FID and the identity of novel peaks confirmed by GC-MS and co-migration with authentic standards.

| Species | Protein ID | Amino Acids | Closest Match on Genbank, % Identity | Designation | Defined Function |
|---|---|---|---|---|---|
| *Ostreococcus* RCC809 | 59992 | 461 | Δ6-desaturase from *O.lucimarinus* (82%) Accession number: DAA34893.1 | **Ost809D6** | C18 Δ6-desaturase |
| *Ostreococcus* RCC809 | 40461 | 459 | Δ4-desaturase from *O.lucimarinus* (85%) Accession number: XP_001415743.1 | **Ost809D4** | C22 Δ4-desaturase |

**Table 3:** LC-PUFA front-end desaturase encoding genes cloned from *Ostreococcus* RCC809.

**BLAST analysis using the amino acid sequence of protein 59992 as a query showed that the protein had high homology to previously reported acyl-CoA Δ6-desaturases from microalgae (**

Figure 11). The protein from *Ostreococcus* RCC809 was most similar to the Δ6-desaturases from its closest relatives, *O. lucimarinus* and *O. tauri* which had an identity of 82% and 75%, respectively. The substrate specificity of the putative Δ6-desaturase was determined by exogenously supplying various substrate fatty acids in the growth media. As shown in Figure 12, heterologous expression of the codon-optimised synthetic ORF encoding *Ostreococcus* RCC809 Protein 59992, predicted to encode a C18 Δ6-desaturase of 461 aa, confirmed the enzymatic capability to convert exogenously supplied substrate α-linolenic acid (ALA; n-3) to the Δ6-desaturated product stearidonic acid (SDA; 18:4n-3). In the absence of galactose, the exogenous substrate ALA is not converted to SDA, since the transgene is not expressed.

```
Ost809D6        -------------------MRVETEDDNVPTVTVGLSEESDGMKGARNPGARAWKSTLEP
O.lucD6         MCVETTEGTSRTMANERTSSSSSLSEGGTPTVTVGMGSE-DAGKKTRNASVTAWTKELEP
O.tauriD6       ------------------MCVETENNDGIPTVEIAFDGE----RERAEANVKLSAEKMEP
M.squaD6        -----------------------------MCPPKESTRKNAGGPLTRGKLSADLAKLEP
M.pusD6         ----------------------------------------MTRGNKAKLDNSKLAKLEP
                                                                       :**

                                                  ▬▬▬▬
Ost809D6        HAVAKSFDRRWVKVDGVEYDVTDFKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKA
O.lucD6         HAIAKTFERRYVTIEGVEYDVTDFKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKA
O.tauriD6       AALAKTFARRYVVIEGVEYDVTDFKHPGGTVIFYALSNTGADATEAFKEFHHRSRKARKA
M.squaD6        HKLAQTFDTRWVRVGDVEYDVTNFKHPGGSVIFYMLSNTGADATEAFNEFHMRSPKAWKM
M.pusD6         HKLAQTFEQRWVRIDDVEYDVTNFKHPGGSVIFYMLSNTGADATEAFKEFHMRSPKAWKM
                  :*::*  *:* : .******:******:**:* ************:*** ** ** *

Ost809D6        LAALPQREPEDAS--PVEDANMLKDFAKWRKDLEREGFFKPSPAHVAYRFAELAAMFALG
O.lucD6         LAALPHKPVDAATREPIEDEAMLKDFAQWRKELEREGFFKPSPAHVAYRFAELAAMFALG
O.tauriD6       LAALPSRP---AKTAKVDDAEMLQDFAKWRKELERDGFFKPSPAHVAYRFAELAAMYALG
M.squaD6        LKALPNRPAETPRSQ-DPDGPMLEDFAKWRAQLEKEGFFKPSIAHVAYRIAELAAMFALG
M.pusD6         LKALPQRPAETPRSA-DPDAPMLQDFARWRAELEKEGFFEPSRLHLAYRCLELCATFALG
                * *** :    .      *  **:***:** :**::***:**  *:***  **.* :***

Ost809D6        TALMYARWHATSVFVTACFFGARCGWVQHEGGHSSLTGSIWWDKRIQAFTAGFGLASSGD
O.lucD6         TALMHARWHVASVIVYSCFFGARCGWVQHEGGHNSLTGNIWWDKRIQAFAAGFGLASSGD
O.tauriD6       TYLMYARYVVSSVLVYACFFGARCGWVQHEGGHSSLTGNIWWDKRIQAFTAGFGLAGSGD
M.squaD6        CYIMSLGYPVVASIVFGAFFGARCGWVQHEGGHNSLTGNIWLDKRIQAATCGFGLSTSGD
M.pusD6         TFLMYIGRPLLASIVYGAFFGARCGWVQHEGGHNSLTGSIWWDKRIQAATCGFGLSTSGD
                  :*       : :* ..***************.****.** ****** :.****: ***

Ost809D6        MWNLMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEENRPRKFSKLWLRVQAWTFVPVTS
O.lucD6         MWNNMHNKHHATPQKVRHDMDLDTTPTVAFFNSAVEENRPRGFSKLWLRLQAWTFVPVTS
O.tauriD6       MWNSMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSKYWLRLQAWTFIPVTS
M.squaD6        MWNQMHNKHHATPQKVRHDMDLDTTPAVAFFKTAVEDNRPRGFSRAWSRAQAWTFVPVTS
M.pusD6         MWNQMHNKHHATPQKVRHDMDLDTTPAVAFFDTAVEDNRPRGFSKTWARAQAWTFVPITS
                *** ********************:****.:***:**** **: * * *****:*:**

Ost809D6        -GLVLLAWMYLLHPRHIARRKNYEEAAWIVAAHVIRTSVIKAVTGYSWITCYGLFLSTMW
O.lucD6         -GMVLFFWMFVLHPRNALRRKSFEEAAWMFSAHVIRTAVIKAVTGYSWIASYGLFAATMW
O.tauriD6       -GLVLLFWMFFLHPSKALKGGKYEELVWMLAAHVIRTWTIKAVTGFTAMQSYGLFLATSW
M.squaD6        GLLVQMFWIYVLHPRQVARKKNYEEASWMILSHVLRTATIKYAGGYSWPVAYLWFSFGNW
M.pusD6         GVLVQMFWIYVLHPRQVLRKKNYEEASWMFLSHVVRTAVIKLALGCGTAEAYGWFWVGNW
                  :* : *::.*** :  :  .:**  *:. :**:** .** . *     .*  *    *

Ost809D6        VSGCYLFAHFSTSHTHLDVVPSDKHLSWVRYAVDHTIDIDPSKSVVNWLMGYLNCQVIHH
O.lucD6         ASGCYLFAHFSTSHTHLDVVPSDKHLSWVRYAVDHTIDINPNNSVVNWLMGYLNCQVIHH
O.tauriD6       VSGCYLFAHFSTSHTHLDVVPADEHLSWVRYAVDHTIDIDPSQGWVNWLMGYLNCQVIHH
M.squaD6        IAYMYLFAHFSTSHTHLEVVPSDKHISWVNYAVDHTVDIDPSKGYVNWLMGYLNCQVIHH
M.pusD6         IAYMYLFAHFSTSHTHLDVVPSDKHISWVNYAVDHTVDINPRNSIVNWLMGYLNCQVIHH
                 :  *************:***:*:*:***.******:**:* :. ***************

Ost809D6        LFPDMPQFRQPEVSRRFVSFAKKWNLNYKVMSYYGAWKATFGNLNEVGKHYYIQGSQITK
O.lucD6         LFPDMPQFRQPEVSRRFVPFAKKWNLNYKVLTYYGAWKATFGNLNDVGKHYYVHGSQRVK
O.tauriD6       LFPSMPQFRQPEVSRRFVAFAKKWNLNYKVMTYAGAWKATLGNLDNVGKHYYVHGQHSGK
M.squaD6        LFPDMPQFRQPEVSRRFVAFAKKWNLNYKVLTYYGAWKATFTNLDTVGQHYYKHGKAHAH
M.pusD6         LFPDMPQFRQPEVSRRFVAFAEKWNLNYKVLTYYGAWKATFSNLDRVGQHYYVNGKAKAH
                ***.**************.**:********::* ******: **: **:*** :*.   :

Ost809D6        KTV-ELGTLI
O.lucD6         SKSA------
O.tauriD6       TA--------
M.squaD6        ----------
M.pusD6         ----------
```

**Figure 11:** Multiple sequence alignment of Ost809D6 with Δ6-desaturases from *Mantoniella squamata* (M.squaD6, CAQ30479.1) [Hoffmann et al, 2008], *Micromonas pusilla* (M.pusD6, XP_002502445.1) (Petrie et al, 2010a), *Ostreococcus lucimarinus* (O.lucD6, DAA34893.1) (Petrie et al, 2010b) and *Ostreococcus tauri* (O.tauriD6, XP_003082578.1) (Domergue et al, 2005). Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of conserved cytochrome b5 domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W (1.83).

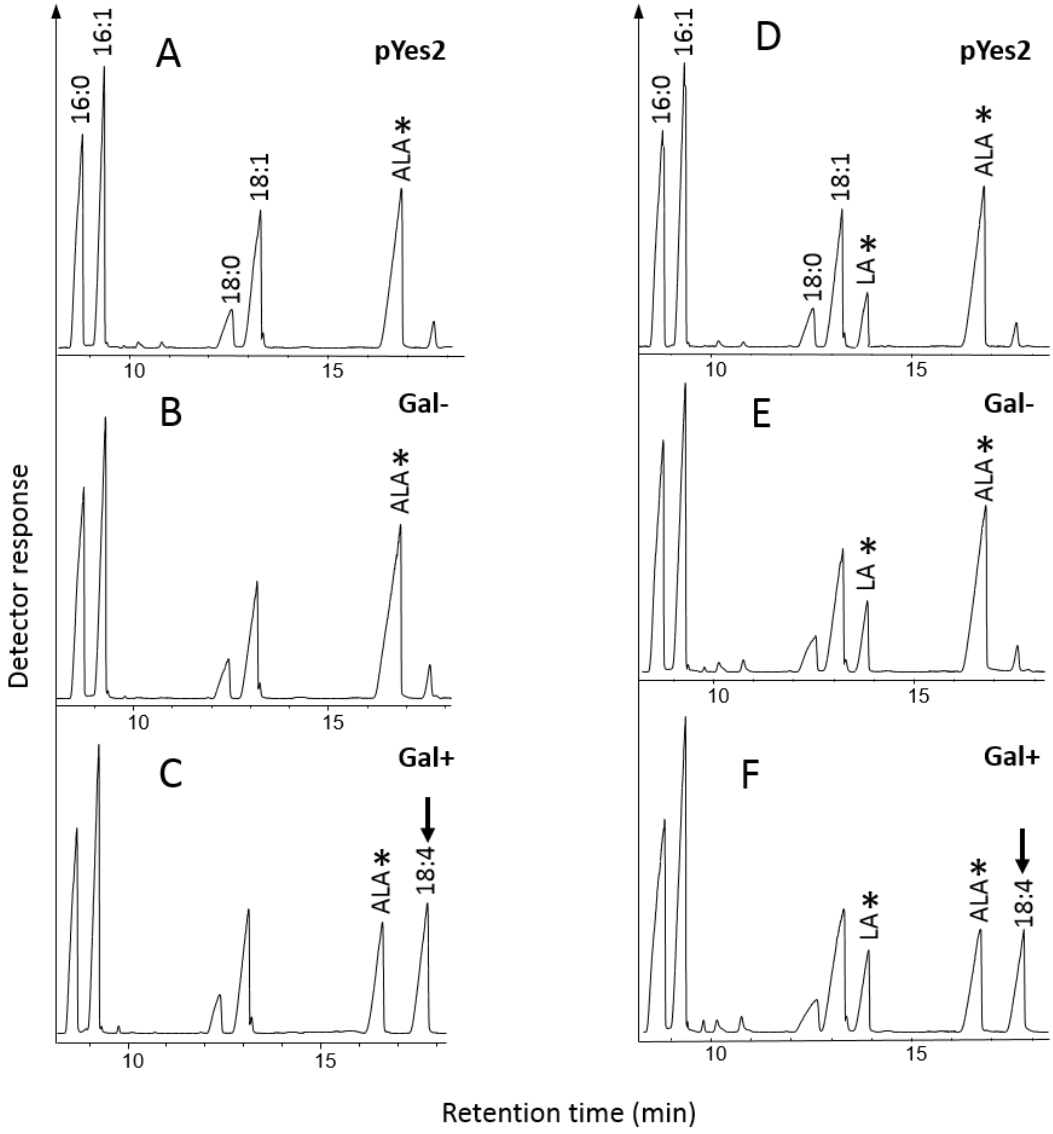



**Figure 12:** Functional characterisation of *Ostreococcus* RCC809 C18 Δ6-desaturase. A synthetic gene encoding *Ostreococcus* RCC809 Protein 59992 was expressed in *S. cerevisiae* under the control of the galactose-inducible GAL promoter in the presence of exogenously supplied substrate α-linolenic acid (ALA) and galactose (C). The presence of the Δ6-desaturation product stearidonic acid (SDA) is indicated (arrowed). In the absence of galactose no conversion of ALA is seen (B). In the presence of galactose and both exogenously supplied ω-3 and ω-6 substrates (ALA and LA respectively) (F), ALA is still converted to SDA but no desaturation of LA is detected. Again, no activity is seen in the absence of galactose (E). The profile of control yeast strain transformed with empty vector is also shown (A and D). The substrate supplemented to the cultures is indicated by an asterisk.

Thus, on the basis of these results, *Ostreococcus* RCC809 Protein 59992 was designated Ost809D6, and represents a new confirmed Δ6-desaturase member of the N-terminal cytochrome b5-fusion desaturase family. Ost809D6 displays high desaturation activity in yeast, converting about 54% of the available substrate ALA, with SDA accumulating to 18.5% of total fatty acids (Table 4). Ost809D6 only recognised the ω-3 fatty acid ALA as a substrate, with the ω-6 substrate LA showing no detectable desaturation in this heterologous yeast expression system (Figure 12). No activity was detected against exogenous 20:3n-6, 20:2n-6, 20:3n-3, 20:4n-3 and 22:5n-3. This strong preference for omega-3 versus omega-6 C18 substrates was also likely reflected in the fatty acid profile of *Ostreococcus* RCC809 (Table 2), with accumulation of omega-6 substrate (LA) but not product (GLA), and the inverse accumulation of omega-3 product (SDA) but not substrate (ALA). This strong omega-3 preference of Ost809D6 is distinct from a Δ6-desaturase with sequence-similarity identified from *O. tauri* (Domergue et al, 2005), which showed high activity towards both LA and ALA as substrates. It is more similar to a Δ6-desaturase identified from *M. squamata* which accepts only ALA as a substrate (Hoffmann et al, 2008). Thus, Ost809D6 is potentially very useful for the exclusive production of Δ6-desaturated omega-3 fatty acids in transgenic plants or algae.

| | Construct | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fatty Acid Composition (molar %) | | | | | | | | | | | |
| **Fatty Acid** | **Ost809 Δ6 Des Gal -** | **Ost809 Δ6 Des Gal +** | **Ost809 Δ6 Des Gal -** | **Ost809 Δ6 Des Gal +** | **Ost809 Δ5 Elo Gal -** | **Ost809 Δ5 Elo Gal +** | **Ost809 Δ5 Elo Gal -** | **Ost809 Δ5 Elo Gal +** | **Frag Δ6 Elo Gal -** | **Frag Δ6 Elo Gal +** | **Ost809 Δ4 Des Gal -** | **Ost809 Δ4 Des Gal +** |
| **16:0** | 25.1 ± 0.3 | 25.4 ± 0.2 | 22.3 ± 0.1 | 22.2 ± 0.4 | 24.3 ± 0.2 | 24.9 ± 0.4 | 26.1 ± 0.4 | 26.3 ± 0.3 | 24.5 ± 0.3 | 22.1 ± 0.1 | 32.8 ± 0.3 | 34.4 ± 0.2 |
| **16:1** | 23.3 ± 0.2 | 26.8 ± 0.4 | 25.4 ± 0.3 | 26.1 ± 0.3 | 29.2 ± 0.3 | 31.1 ± 0.5 | 33.4 ± 0.4 | 29.8 ± 0.2 | 26.8 ± 0.2 | 25.1 ± 0.3 | 38.9 ± 0.4 | 37.8 ± 0.3 |
| **18:0** | 3.5 ± 0.4 | 3.7 ± 0.3 | 3.7 ± 0.5 | 3.3 ± 0.3 | 4.3 ± 0.5 | 3.8 ± 0.2 | 3.5 ± 0.1 | 3.4 ± 0.2 | 3.7 ± 0.4 | 3.2 ± 0.3 | 7.9 ± 0.4 | 7.3 ± 0.5 |
| **18:1** | 15.2 ± 0.5 | 15.5 ± 0.3 | 13.4 ± 0.4 | 14.2 ± 0.4 | 20.8 ± 0.3 | 21.2 ± 0.4 | 19.8 ± 0.1 | 18.1 ± 0.2 | 15.3 ± 0.3 | 13.2 ± 0.5 | 17.8 ± 0.3 | 17.2 ± 0.4 |
| **18:2** | **5.7 ± 0.2** | **6.4 ± 0.1** | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| **GLA** | ND | ND | ND | ND | ND | ND | ND | ND | **29.7 ± 0.2** | **22.3 ± 0.1** | ND | ND |
| **ALA** | **25.6 ± 0.1** | **11.9 ± 0.1** | **32.9 ± 0.2** | **15.7 ± 0.1** | ND | ND | ND | ND | ND | ND | ND | ND |
| **SDA** | 1.6 ± 0.3 | 10.3 ± 0.2 | 2.3 ± 0.6 | 18.5 ± 0.1 | ND | ND | ND | ND | ND | ND | ND | ND |
| **DHGLA** | ND | ND | ND | ND | ND | ND | ND | ND | ND | 14.1 ± 0.2 | ND | ND |
| **ARA** | ND | ND | ND | ND | **21.4 ± 0.2** | **9.2 ± 0.3** | ND | ND | ND | ND | ND | ND |
| **EPA** | ND | ND | ND | ND | ND | ND | **17.2 ± 0.6** | **8.5 ± 0.5** | ND | ND | ND | ND |
| **DTA** | ND | ND | ND | ND | ND | 9.8 ± 0.5 | ND | ND | ND | ND | ND | ND |
| **DPAn-6** | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | **2.6 ± 0.3** | **2.8 ± 0.2** |
| **DPAn-3** | ND | ND | ND | ND | ND | ND | ND | 13.9 ± 0.2 | ND | ND | ND | ND |
| **DHA** | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | 0.5 ± 0.2 |

**Table 4:** Fatty acid composition (%) of transgenic yeast. Values are the average of three independent experiments ± standard deviation. ND—not detected. The substrate supplemented to the culture is indicated in bold.

#### 2.3.1.3 Identification and Functional Characterisation in Yeast of a Putative Δ4-Desaturase from *Ostreococcus* RCC809

As described above, the genome sequence of *Ostreococcus* RCC809 was searched with previously functionally characterised sequences of Δ4-desaturases and the presence of an ortholog (JGI protein ID # 40461) for a Δ4-desaturase was detected (Table 3). The deduced amino acid sequence was used as a query for similarity searches using BLAST analyses after which a multiple alignment was created (Figure 13). The most similar proteins were Δ4-desaturase from *O. lucimarinus* and chloroplast Δ6- desaturase from *C. reinhardtii* which had identities of 85% and 41%, respectively, although the protein demonstrated very low similarity to previously reported Δ4-desaturases (Meyer et al, 2004; Tonon et al, 2005a; Petrie et al, 2010b; Sayanova et al, 2011). The deduced open reading frame was again used as a template to chemically synthesise codon-optimised nucleotide sequences for expression in the diatom *T. pseudonana*. The synthetic ORF of the putative Δ4-desaturase was inserted as a KpnI-SacI fragment behind the galactose-inducible GAL1 promoter of the yeast expression vector pYES2 and was tested for activity against the appropriate 22:5n-3 (DPA) substrate (Figure 14). Expression of the synthetic predicted ORF encoding a polypeptide of 459 amino acids resulted in the Δ4-desaturation of DPA to DHA, with a conversion rate of 15.1% (0.5% accumulation of DHA, Table 4), confirming the function of this ORF as a C22 Δ4-desaturase and on this basis this protein was designated as Ost809D4. Note that in the absence of the inducer (galactose), no DHA is detected, nor in the absence of the Ost809D4 ORF. No activity was detected against exogenously supplied potential substrate for Δ5-desaturation, 20:3n-6 (DGLA) (data not shown). Thus, although *Ostreococcus* RCC809 synthesises only limited levels of DHA, its genome encodes a fully functional enzyme for the terminal desaturation step in the biosynthesis of DHA. In that respect, it may be that under some particular environmental conditions or lifecycle stages this gene is more actively expressed and higher levels of DHA are generated.

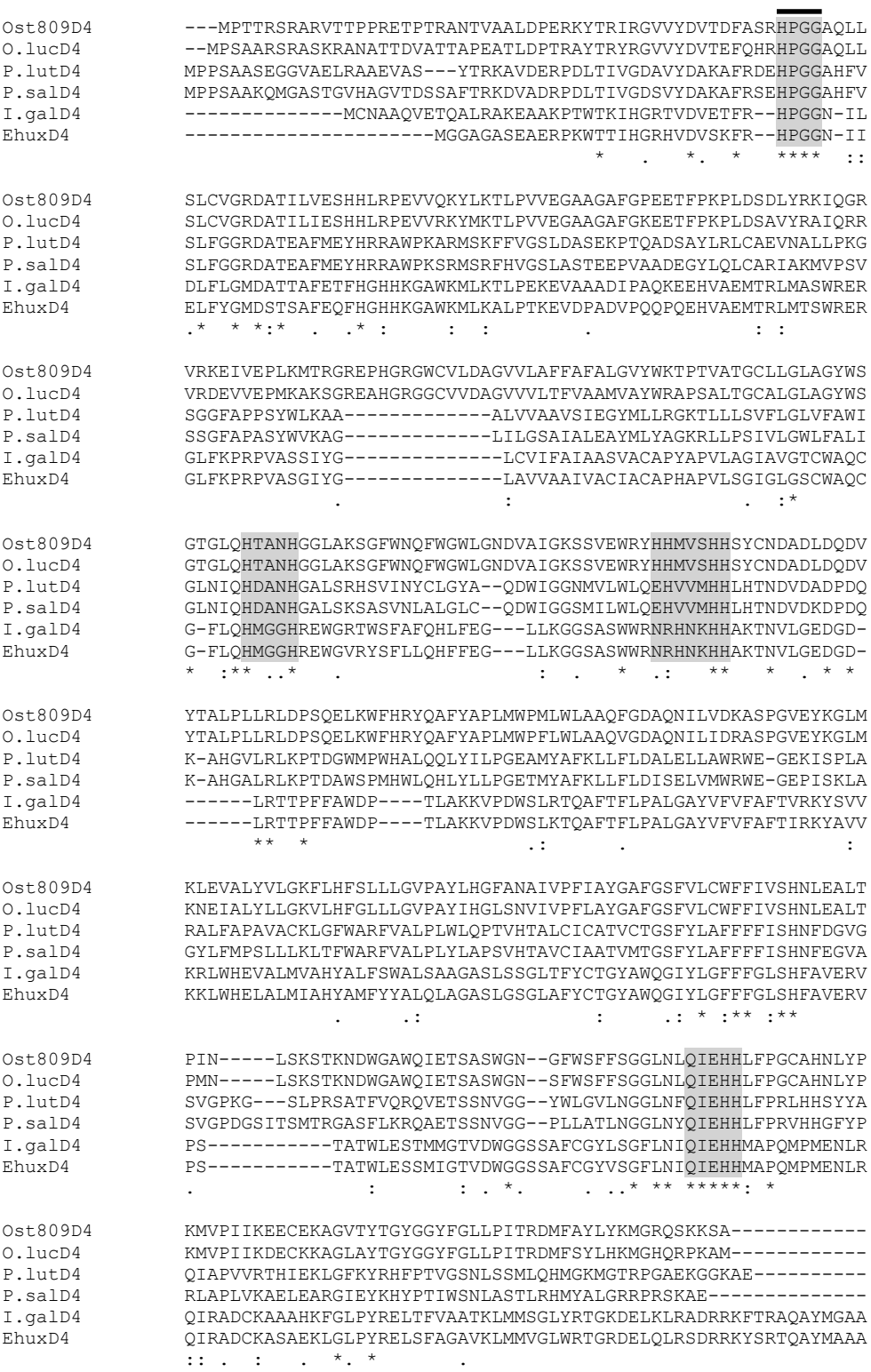



**Figure 13:** Multiple sequence alignment of Ost809D4 with Δ4-desaturases from *Pavlova lutheri* (P.lutD4, AAQ98793.1), *Pavlova salina* (P.salD4, AY926606.1) (Domergue et al, 2005), Isochrysis galbana (I.galD4, AY630574) (Meyer et al, 2004), *Ostreococcus lucimarinus* (O.lucD4, XM_001415706.1) (Ahman et al, 2011) and *Emiliania huxleyi* (E.huxD4) (Sayanova et al, 2007). Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of the conserved cytochrome b5 domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W (1.83). A related Δ4-desaturase from *Thalassiosira pseudonana* (TpD4, AAX14506.1) (Tonon et al, 2005a) was omitted from this line-up on the basis of poor alignment.

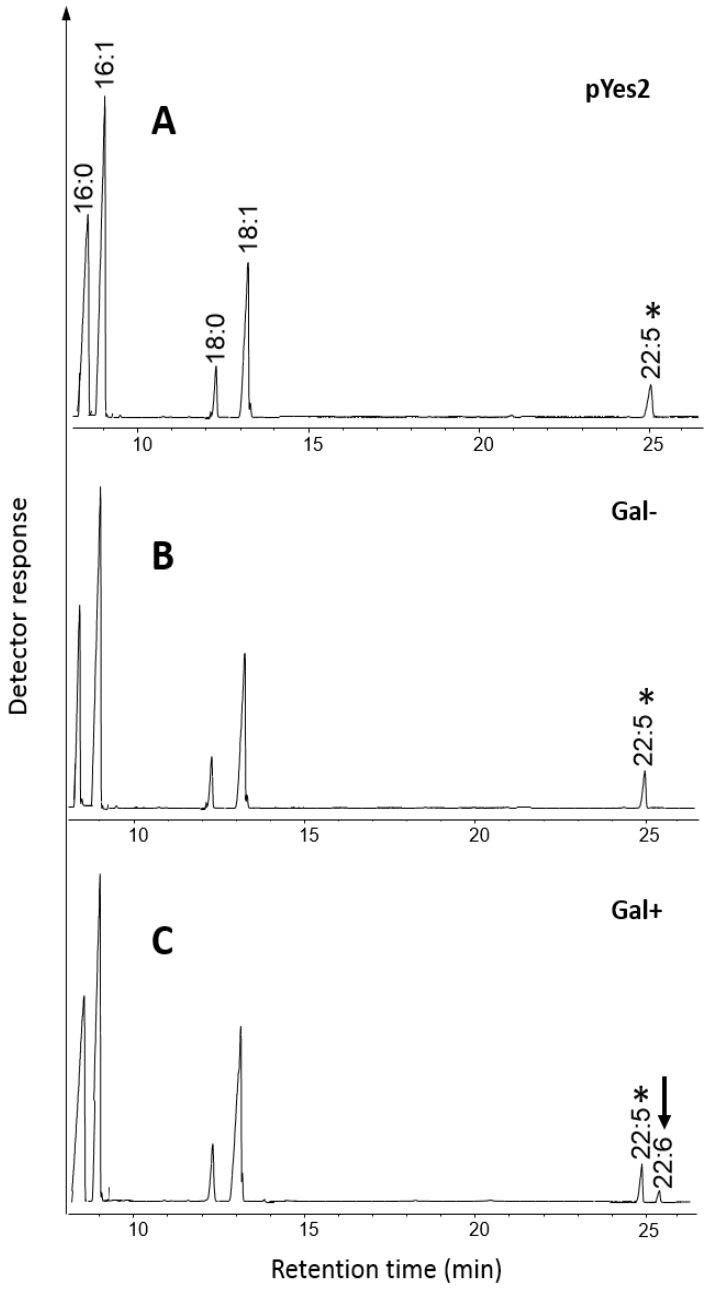



**Figure 14:** Functional characterisation of *Ostreococcus* RCC809 C22 Δ4-desaturase. In the presence of exogenously supplied substrate (22:5n-3) and galactose, the accumulation of the Δ4-desaturation product DHA is detected (C) when total yeast fatty acids are analysed. In the absence of galactose, no conversion of 22:5n-3 is seen (B). The profile of control yeast strain transformed with empty vector is also shown (A). The substrate supplemented to the cultures is indicated by an asterisk.

#### 2.3.1.4 Identification and Functional Characterisation in Yeast of a Putative Δ5-Elongase from *Ostreococcus* RCC809

As described above, the genome sequence of *Ostreococcus* RCC809 was searched with previously functionally characterised sequences of Δ5-elongases and the presence of an ortholog (JGI protein ID # 36819) for a Δ5-elongase was detected. Similarity searches were undertaken to produce a multiple alignment (Figure 15). The most similar proteins were a predicted elongase protein from *O. lucimarinus* (*Ostreococcus lucimarinus* CCE9901, XP_001419791.1) and a Δ5-elongase from *O. tauri* (OtElo2, AAV67798.1), with identities of 84% and 80%, respectively. This putative elongase displayed very low similarity to previously reported Δ5-elongases (Meyer et al, 2004; Robert et al, 2008; Niu et al, 2009).

```
Ost809Elo5       -MTRVPVVALVAFAVYGYATYAYATDWSHARTGG-MANVNVQQWIGDLSLALPACATSGY
O.lucPutElo      -MAQFPLVSLCAFAVYGYATYAYAFEWSHARTPGGLANVDAQRWIGDLSFALPACATTAY
O.tauriElo2      MSASGALLPAIAFAAYAYATYAYAFEWSHANG---IDNVDAREWIGALSLRLPAIATTMY
                   :  .::.  ***.*.******* :****.    : **:.:.*** **: *** **: *

Ost809Elo5       LLFCLLGPRVMASREAMDPKGFMLAYNAYQTVFNVGVLGLFIHEIIALKQPMWGSKLPWS
O.lucPutElo      LMFCLVGPRVMAKREAFDPKGLMLAYNAYQTAFNVCVLGMFIREIVTLKQPTWGSKMPWS
O.tauriElo2      LLFCLVGPRLMAKREAFDPKGFMLAYNAYQTAFNVVVLGMFAREISGLGQPVWGSTMPWS
                 *:***:***:**.***:****:*********.*** ***:* :**  * ** ***.:***

Ost809Elo5       DNRTFKLLLGVWFHYNNKYLELLDTVFMVVRKKTKQLSFLHVYHHALLIWAWWFVCHLMA
O.lucPutElo      DKRSFNILLGVWFHYNNKYLELLDTAFMIARKKTNQLSFLHVYHHALLIWAWWFVCHLMA
O.tauriElo2      DRKSFKILLGVWLHYNNQYLELLDTVFMVARKKTKQLSFLHVYHHALLIWAWWLVCHLMA
                 *.::*::*****:****:*******.**:.****:******************:******

Ost809Elo5       RNDCADAYFGAACNSFIHIVMYSYYLMAALGISCPWKRYITQAQMLQFVVVFVHAVFVLR
O.lucPutElo      TNDCVDAYFGAACNSFIHIVMYSYYLMAALGVRCPWKRYITQAQMLQFVIVFVHAVFVLR
O.tauriElo2      TNDCIDAYFGAACNSFIHIVMYSYYLMSALGIRCPWKRYITQAQMLQFVIVFAHAVFVLR
                  *** **********************:***: ****************:**.*******

Ost809Elo5       EQHCPVSLPWAQMFVMANMLVLFGNFYLKAYASKTSA-AAPKKPPTTTTRAPSTRRTRSR
O.lucPutElo      EKHCPVSLPWAQMFVMANMLVLFGNFYLKAYAAKPSG-KSSTR---VSAAKPATRRTRSR
O.tauriElo2      QKHCPVTLPWAQMFVMTNMLVLFGNFYLKAYSNKSRGDGASSVKPAETTRAPSVRRTRSR
                 ::****:*********:**************: *. .  :..     ::  *:.******

Ost809Elo5       KVD
O.lucPutElo      KID
O.tauriElo2      KID
                 *:*
```

**Figure 15:** Multiple sequence alignment of Ost809Elo5 with a putative elongase from *O. lucimarinus* (O. lucPutElo, XP_001419791.1) and a Δ5-elongase from *O. tauri* (O.tauriElo2, XP_001419791.1). Conserved amino acid residues are indicated with an asterisk, whereas conserved motifs are shaded. The alignment was obtained using CLUSTAL W (1.83). Previously reported elongases (Meyer et al, 2004; Robert et al, 2008; Niu et al, 2009) were omitted due to poor alignment.

To confirm the function of this putative elongase, the deduced ORF was again used as a template to chemically synthesise codon-optimised nucleotide sequences for expression in the diatom *T. pseudonana*, and functionally characterised in yeast in the presence of exogenously supplied substrates ARA and EPA. Expression of the synthetic predicted ORF encoding a polypeptide of 301 amino acids resulted in the Δ5-elongation of ARA to 22:4n-6

(DTA) (Figure 16) and Δ5-elongation of EPA to 22:5n-3 (DPA) (Figure 16), with a conversion rate of 51.6% (Table 4), confirming the function of this ORF as a C20 Δ5-elongase. On this basis this protein was designated as Ost809Elo5. Ost809Elo5 elongated ω-3 substrate EPA with a higher conversion rate of 62.1% (Table 4). No elongation was observed with other exogenously added potential substrates that were tested (data not shown).

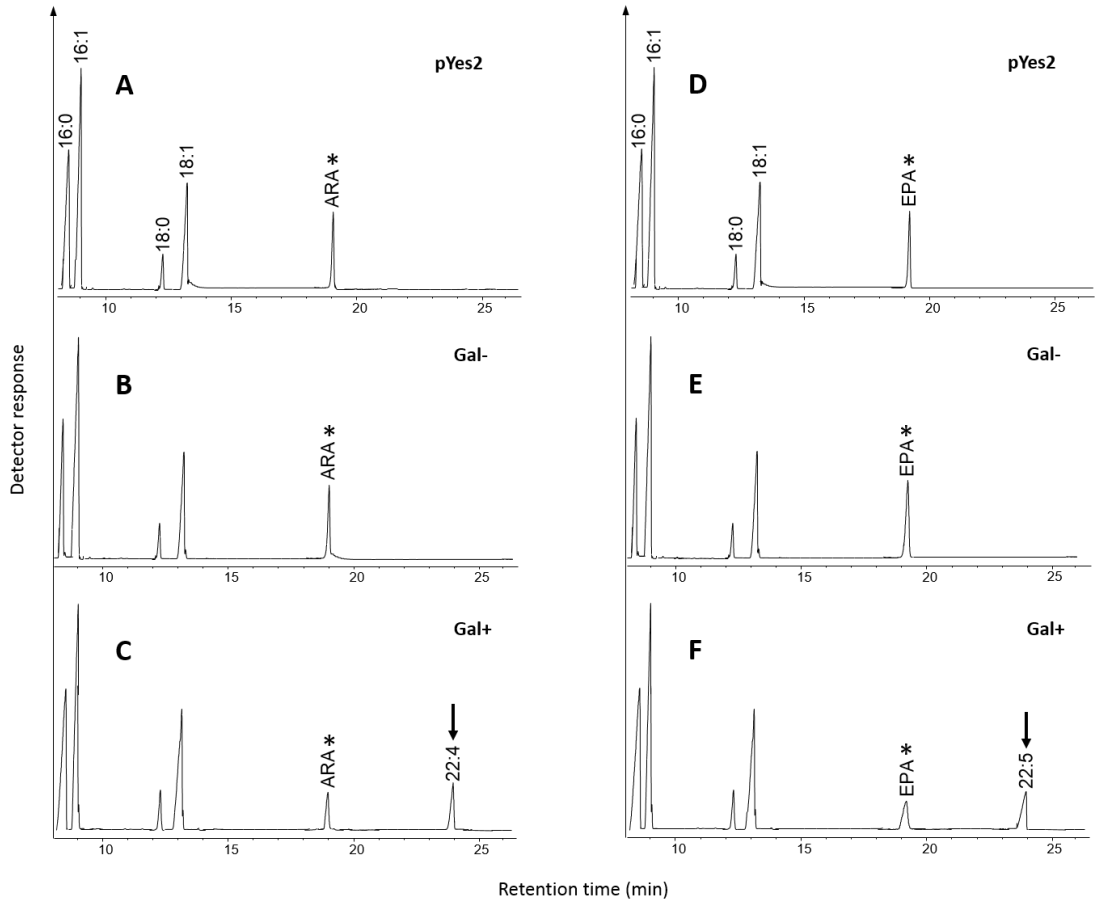



**Figure 16:** Functional characterisation of *Ostreococcus* RCC809 C20 Δ5-elongase. In the presence of exogenously supplied ω-6 substrate (ARA) and galactose, the accumulation of the Δ5-elongation product DTA is detected (C) when total yeast fatty acids are analysed. In the presence of exogenously supplied ω-3 substrate (EPA) and galactose, the accumulation of the Δ5-elongation product DPA is detected (F). In the absence of galactose, no conversion of either substrate is seen (B and E). The profile of control yeast strain transformed with empty vector is also shown (A and D). The substrate supplemented to the cultures is indicated by an asterisk.

## 2.3.2 Identification and Functional Characterisation of a Putative Δ6-Elongase from *Fragilariopsis cylindrus*

### 2.3.2.1 Fatty Acid Composition of *Fragilariopsis cylindrus*

The GC-FID analysis of FAMEs of total lipids from stationary phase cultures of *F. cylindrus* (Figure 17) revealed that the most abundant fatty acid in this diatom was EPA (31.4% of TFA) followed by 16:1 and 16:0 (24.5% and 12.1% respectively) (Table 2). Similarly to *Ostreococcus* RCC809, only low levels of DHA were observed, although SDA levels were markedly lower in *F. cylindrus*. On the basis of significant levels of the C20 fatty acid EPA, it was indicative that this diatom contains a Δ6-elongase activity capable of elongating SDA to ETA prior to Δ5-desaturation, and for this reason, efforts were focused on identifying this gene.

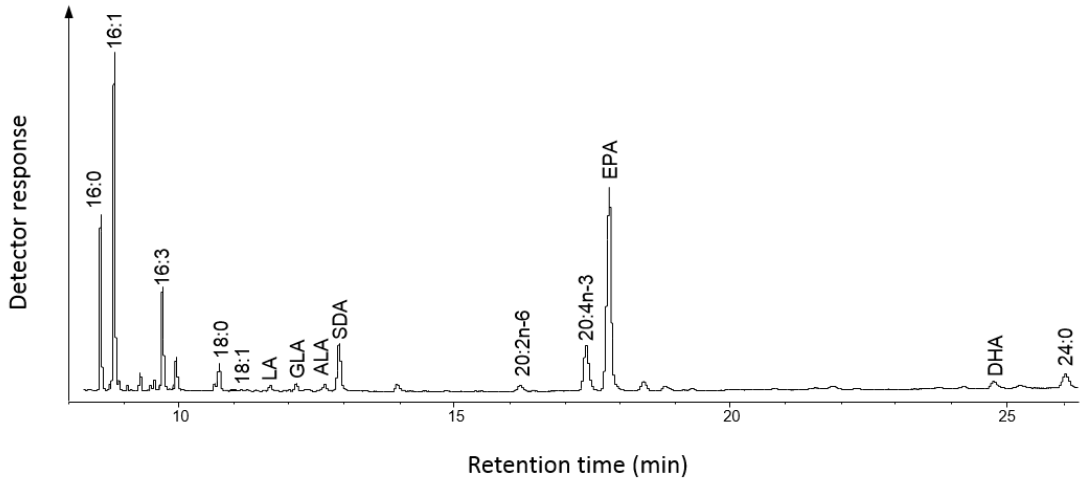



**Figure 17:** Total fatty acid methyl esters of *F. cylindrus*. FAMEs were prepared and analysed by GC-FID, with peaks being identified by co-migration against known standards. The identity of major peaks is shown.

### 2.3.2.2 Identification and Functional Expression in Yeast of a Putative Elongase

Using a similar approach to that used for *Ostreococcus* RCC809, the publicly available genome sequence of the marine diatom *F. cylindrus* (Joint Genome Institute *Fragilariopsis cylindrus* genome project) was probed via a BLAST search interface, using previously characterised Δ6-elongase sequences as the query template. This identified one strong candidate, and the deduced ORF of 287 amino acids (designated Frag#177742) was used as a query for similarity searches. Frag#177742 showed the highest identity (70%) to Δ6-elongases from diatoms, *T. pseudonana* (Meyer et al, 2004) and *P. tricornutum* (Accession number AAW70157) (Figure 18).

```
FcElo6          ----------------------MDEYKATLESVGDAIIQWADPESQFTGFTKGWFLTDFTS
TpElo6          ----------------------MDAYNAAMDKIGAAIIDWSDPDGKFRADREDWWLCDFRS
OtElo6          MSGLRAPNFLHRFWTKWDYAISKVVFTCADSFQWDIGPVSSSTAHLPAIESPTPLVTSLL
                                       .   :  :..   *   :.. .:: .  .   *

FcElo6          AFSIALVYVLFVIIGSQVMKVLPAIDPYPIKFFYNVSQIMLCAYMTIEACLLAYRNGYTI
TpElo6          AITIALIYIAFVILGSAVMQSLPAMDPYPIKFLYNVSQIFLCAYMTVEAGFLAYRNGYTV
OtElo6          FYLVTVFLWYGRLTRSSDKKIREPTWLRRFIICHNAFLIVLSLYMCLGCVAQAYQNGYTL
                   :::.     :  *   :   .     : : :*.  *.*. ** : .   **:****:

FcElo6          MPCVGYNRDDPAIGNLLWLFYVSKVWDFWDTIFIVLGKKWRQLSFLHVYHHTTIFLFYWL
TpElo6          MPCNHFNVNDPPVANLLWLFYISKVWDFWDTIFIVLGKKWRQLSFLHVYHHTTIFLFYWL
OtElo6          WG-NEFKATETQLALYIYIFYVSKIYEFVDTYIMLLKNNLRQVSFLHIYHHSTISFIWWI
                     ::  :. :.  :::**:**:::* ** :::* :: **:****:***:** :::*:

FcElo6          NANVFYDGDIYLTIALNGFIHTVMYTYYFICMHTKDKKTGKSLPIWWKSSLTLLQLFQFI
TpElo6          NANVLYDGDIFLTILLNGFIHTVMYTYYFICMHTKDSKTGKSLPIWWKSSLTAFQLLQFT
OtElo6          IARRAPGGDAYFSAALNSWVHVCMYTYYLLSTLIGKEDPKRSNYLWWGRHLTQMQMLQFF
                 *.   .** :::  **.::*. *****::.    .... :*  :**   ** :*::**

FcElo6          TMMSQGLYLIIFGCESLSIRVTATYVVYILSLFFLFAQFFVASYMQPKKSKTA-ELGTLI
TpElo6          IMMSQATYLVFHGCDKVSLRITIVYFVSLLSLFFLFAQFFVQSYMAPKKKKSA-------
OtElo6          FNVLQALYCASF--STYPKFLSKILLVYMMSLLGLFGHFYYSKHIAAAKLQKKQQ-----
                  : *. *   .  .. .  ::   .* ::**: **.:*:  .:: . * :.
```

**Figure 18:** Multiple sequence alignment of FcElo6 with Δ6-elongases from *Thalassiosira pseudonana* (TpElo6, AY591337.1) and *Ostreococcus tauri* (OtElo6, AY591335) (Meyer et al, 2004). Conserved amino acid residues are indicated with an asterisk, whereas conserved motifs are framed. The alignment was obtained using CLUSTAL W (1.83).

To confirm the function of this putative elongase sequence, the synthetic ORF was expressed in yeast in the presence of exogenous 18:3n-6, GLA (Figure 19). Expression of Frag#177742 in yeast demonstrated that this sequence directed the elongation of GLA to generate 20:3n-6, DGLA (14.1% of TFA, 38.1% conversion rate). Thus, Frag#177742 was redesignated FcElo6, on the basis of possessing *bona fide* elongating activity specific for C18 Δ6-unsaturated substrates. The substrate specificity of FcElo6 was analysed by exogenously supplying equal quantities of GLA and SDA in the growth media (Figure 20). As shown in Table 4, FcElo6 elongated SDA to generate 20:4n-3 at similarly high proportions (39% conversion rate). No elongation was observed with other exogenously added potential substrates that were tested (LA and EPA).

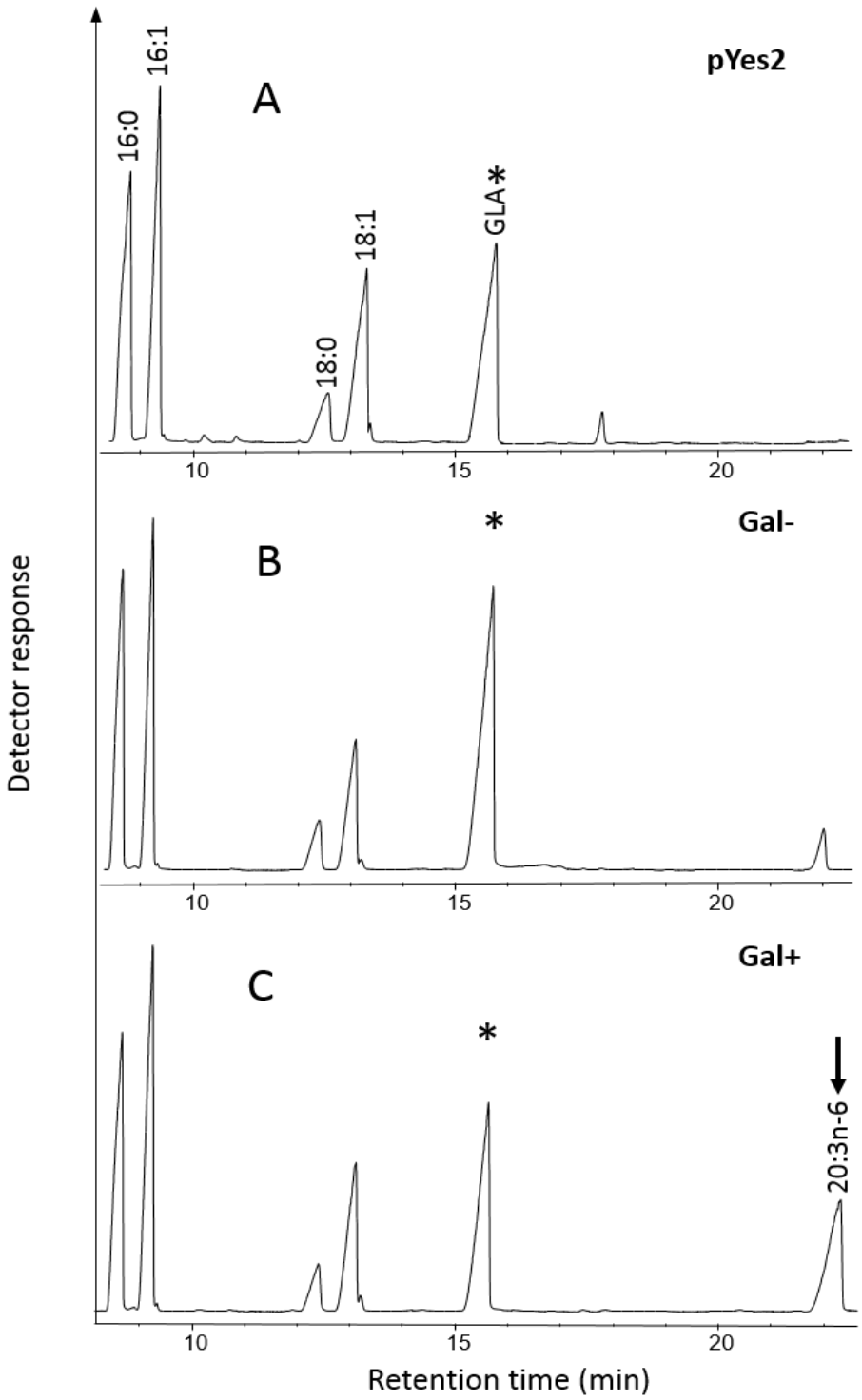



**Figure 19:** Functional characterisation of *F. cylindrus* C18 Δ6-elongase. A synthetic gene encoding *F. cylindrus* Protein 177742 was expressed in *S. cerevisiae* under the control of the galactose-inducible GAL promoter. In the presence of exogenously supplied substrate (GLA) and galactose, the presence of the elongation product 20:3n-6 is detected when total yeast fatty acids are analysed (C). In the absence of galactose, no elongation of GLA is seen (B). The profile of control yeast strain transformed with empty vector is also shown (A). The substrate supplemented to the cultures is indicated by an asterisk. Arrow indicates additional peak corresponding to DGLA, 20:3n-6.

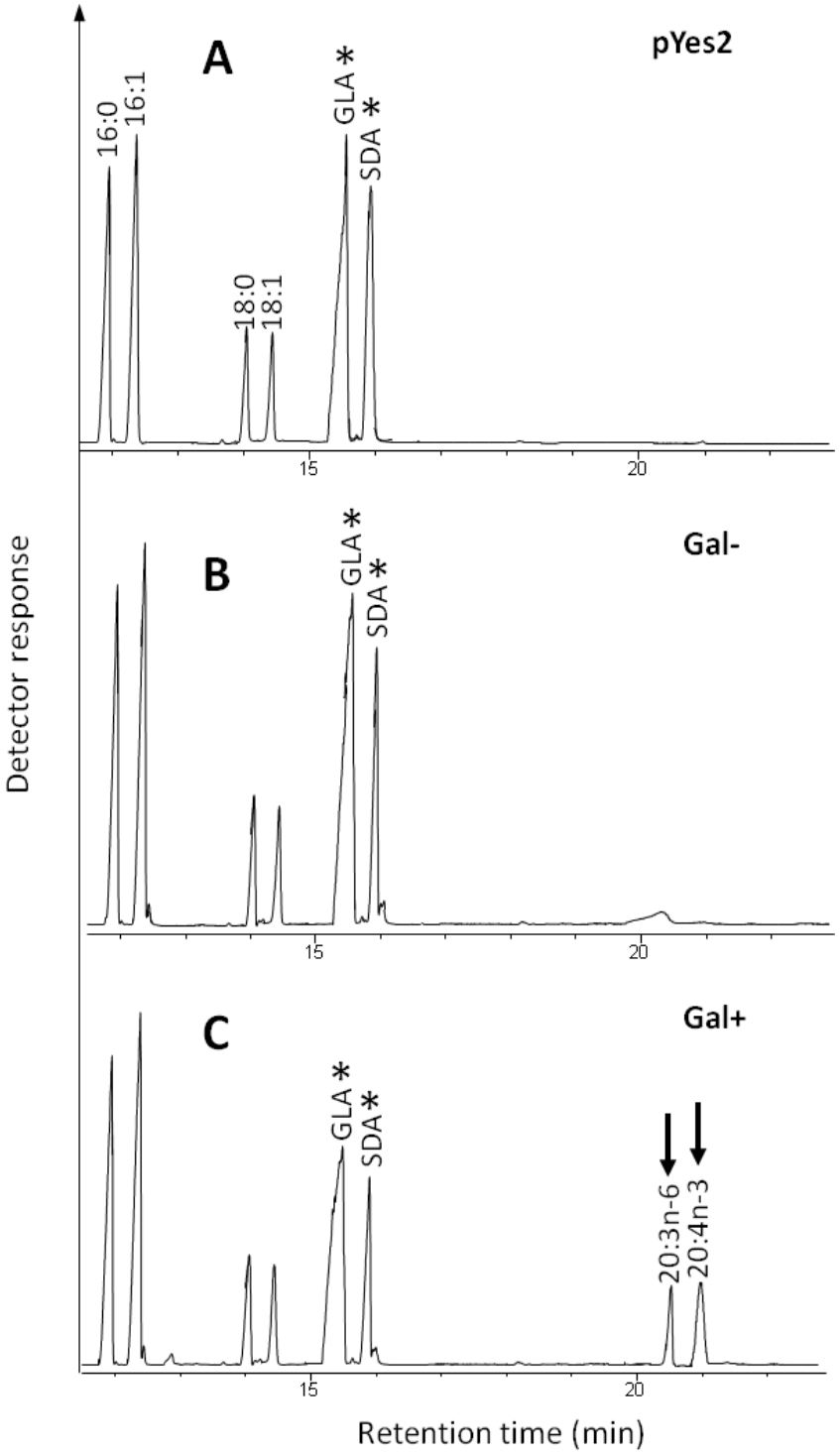



**Figure 20:** Functional characterisation of *F. cylindrus* C18 Δ6-elongase. *S. cerevisiae* cultures containing Protein 177742 under the control of the galactose-inducible GAL promoter were also tested in the presence of both exogenously supplied GLA and SDA. In the presence of galactose the elongation products 20:3n-6 and 20:4n-3 are both detected when total yeast fatty acids are analysed (C). In the absence of galactose, no elongation of GLA is seen (B). The profile of control yeast strain transformed with empty vector is also shown (A). The substrate supplemented to the cultures is indicated by an asterisk. The arrow indicates the additional peaks corresponding to the elongation products DGLA, 20:3n-6 and ETA, 20:4n-3.

## 2.4 CONCLUSIONS

In conclusion, through a database search of the genomes of two primary producers of LC-PUFAs, four novel genes involved in the biosynthesis of the nutritionally important omega-3 polyunsaturated fatty acids in marine microalgae have been identified and functionally characterised. Of particular interest is the Δ6-desaturase from the unicellular photoautotrophic green alga *Ostreococcus* RCC809, which demonstrated activity with specificity towards omega-3 substrates, making this an interesting candidate for heterologous expression in transgenic plants. Previous studies (Sayanova et al, 2003; Sayanova et al, 2006) have identified unusual higher plant Δ6-desaturases from the *Primulaceae*, which showed distinct preferences for either omega-3 or omega-6 C18 substrates. Similar to the results presented above, it was not possible to identify the precise amino acid determinants of such specificities, despite a high degree of similarity between enzymes with different substrate preferences. The *Ostreococcus* RCC809 activity described here is another example of an algal desaturase with strong selectivity for n-3 substrates and better conversion rates of the n-3 substrate ALA than for previously reported Δ6-desaturases from microalgae (Hoffmann et al, 2008; Petrie et al, 2010a; Petrie et al, 2010b). One additional factor which remains to be determined for this particular enzyme is the nature of substrate used by the desaturase. One of the most closely related orthologs of Ost809D6 is the Δ6-desaturase from *Ostreococcus tauri* (Figure 11), which has been shown to prefer acyl-CoA substrates as opposed to the phospholipid-linked substrates more normally associated with lower eukaryotic desaturation (Domergue et al, 2005). Based on the above results, it is not possible to infer any such tendency in the *Ostreococcus* RCC809 desaturase, but the definitive experiments remain to be carried out. Collectively, the algal genes functionally characterised above further add to our understanding of the biosynthesis of the vital and valuable omega-3 LC-PUFAs in the marine food-web, and also provide additional molecular tools with which to attempt the heterologous reconstruction of that biosynthetic pathway in transgenic hosts such as plants, as well as for overexpression studies in other algal species.

# Chapter 3

# Overexpression Of Genes Involved In Omega-3 Biosynthesis In The Diatom *Thalassiosira Pseudonana*

## 3.1 ABSTRACT

In order to enhance the production of EPA and DHA omega-3 polyunsaturated fatty acids in the marine diatom *Thalassiosira pseudonana*, genes from other algal species encoding omega-3 biosynthetic activities were overexpressed in *T. pseudonana*. These heterologous genes encoding two Δ6-desaturases, a Δ6-elongase and two Δ5-elongases were transformed under the control of a constitutive promoter. Preliminary results revealed markedly increased SDA, EPA and DHA production, particularly for one of the transgenic lines overexpressing a Δ6-desaturase. Further analyses over two years revealed an almost cyclic variability to the FAMEs profiles, but not a seasonal rhythm. Interestingly, significant variation was also observed in wild type fatty acid composition during the same periods, therefore skewing the magnitude of enhancement of omega-3 PUFAs observed in the transgenic lines. Acyl-CoA analysis was carried out on the mutant lines from each overexpression series, revealing that transgenic lines contained larger pools of longer chain acyl-CoAs compared to wild type. Microscopic analyses of the same transgenic lines revealed altered lipid body formation and distribution, as well as altered chloroplast morphology. These results suggest that the overexpression of these genes in *T. pseudonana* has affected lipid metabolism, although other factors may be involved in maintaining the enhanced production of targeted fatty acids. Furthermore, the cyclic variation observed in wild type FAMEs profiles over time suggests clock mechanisms may be a contributing factor in the control of lipid metabolism in this species. The periodicity could be related to the cells needing to store more TAG during winter periods, suggesting an endogenous anticipatory clock mechanism that is independent of external cues.

## 3.2 INTRODUCTION

As discussed in depth in Chapter 1, a promising and viable alternative source of omega-3 polyunsaturated fatty acids is the metabolic engineering of microalgal species to increase their endogenous level of PUFA production and subsequent incorporation into TAG. *Thalassiosira pseudonana* produces both EPA and DHA through the conventional Δ6-desaturase pathway discussed in Chapter 2. EPA and DHA in *T. pseudonana* are partitioned into TAG during the stationary phase of growth (Tonon et al, 2002). The native genes encoding Δ5-elongase and Δ6-, Δ5- and Δ4-desaturases involved in the biosynthesis of EPA and DHA in *T. pseudonana* have been identified (Pereira et al, 2004; Tonon et al, 2005a).

In order to enhance and redirect endogenous production of these valuable omega-3 fatty acids, heterologous genes encoding two Δ6-desaturases, a Δ6-elongase and two Δ5-elongases (from *Ostreococcus tauri* and *Ostreococcus* sp. RCC809, *Fragilariopsis cylindrus*, and *Ostreococcus tauri* and *Ostreococcus* sp. RCC809, respectively) were transformed into *T. pseudonana* under the control of a constitutive promoter. The *O. tauri* Δ6-desaturase was transformed as a GFP fusion construct to confirm the presence and location of the transgenic protein through imaging studies. The identification and functional characterisation of three of these genes was discussed in Chapter 2 and this data has since been published and used as the basis of a patent (Vaezi et al, 2013; Napier et al, 2014). The functionalities of the *O. tauri* Δ6-desaturase and Δ5-elongase have been previously characterised (Domergue et al, 2005 and Petrie et al, 2010b, respectively).

Fatty acid profiling using GC-FID and GC-MS to examine fatty acid methyl esters (FAMEs) was used to determine the effect of overexpression of these genes on the fatty acid composition of *T. pseudonana*. Variations were detected, with increased EPA and DHA synthesis observed a number of times, but these FAMEs profiles were not consistent throughout the course of this PhD. The compositions of acyl-CoA pools in transgenic and wild type lines were also analysed. Microscopy studies were carried out on transgenic and wild type lines to assess the occurrence of any phenotypic changes associated with the overexpression of each gene, as well as to confirm the presence and location of the transgenic protein in the case of the *O.tauri* Δ6-desaturase. These imaging studies revealed marked phenotypic variation in the transgenic lines.

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Generation of transgenic *T. pseudonana* and development of an optimised colony PCR method for the identification of positive transformants

Since *T. pseudonana* synthesises EPA and DHA through the conventional Δ6-desaturase pathway (Tonon et al, 2002) heterologous genes encoding these biosynthetic activities were chosen for overexpression studies in this organism. These genes are shown in Table 5. They were codon optimised for expression in *T. pseudonana* and cloned into the pTpFCP-GFP vector and co-transformed (Figure 21) with a plasmid conferring resistance to nourseothricin.

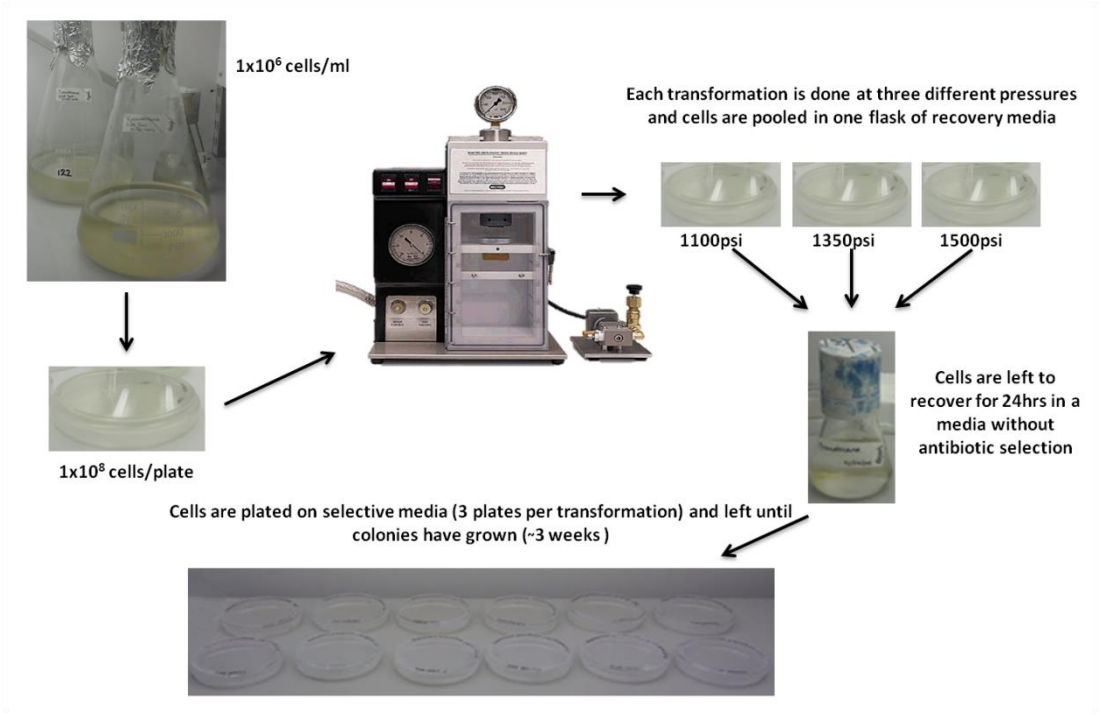



**Figure 21:** Biolistic transformation protocol for *T. pseudonana* as developed by Poulsen et al, 2006.

Table 5 shows the results after numerous transformation experiments. Viable Tp_O809D6 and Tp_O809Elo5 colonies could not be obtained, despite repeated experiments. Selected nourseothricin-resistant colonies from successful transformation experiments were transferred into liquid ESAW medium and grown under constant illumination (100 µmol photons $m^{-2}$ $s^{-1}$) and temperature (20°C).

| Construct | Activity Encoded by Transgene | Source Organism | Transformed into *T. pseudonana* | Number of Colonies | Transgenic Line |
|---|---|---|---|---|---|
| pTp_OtD6GFP | Δ6-desaturase | *O. tauri* (Tonon et al, 2005a) | ✓ | >5 | Tp_OtD6GFP |
| pTp_O809D6 | Δ6-desaturase | *Ostreococcus* sp. RCC809 (Vaezi et al, 2013) | × | 0 | Tp_O809D6 |
| pTp_FragElo6 | Δ6-elongase | *F. cylindrus* (Vaezi et al, 2013) | ✓ | >5 | Tp_FragElo6 |
| pTp_OtElo5 | Δ5-elongase | *O. tauri* (Pereira et al, 2004 | ✓ | >10 | Tp_OtElo5 |
| pTp_O809Elo5 | Δ5-elongase | *Ostreococcus* sp. RCC809 (Chapter 2) | × | 0 | Tp_O809Elo5 |

**Table 5:** List of constructs transformed into *T. pseudonana*, origin of transgene and the transformation results.

In order to confirm the presence of the transgene a sample was taken from each culture and screened using an optimised "colony PCR" method based on DNA extraction using Chelex-100 (Bio-Rad Laboratories, Richmond, CA) (Walsh et al, 1991). A number of variations on this method were experimented with until an optimal and reproducible protocol was found. Each culture was screened as per this final protocol. A 10ml sample from a stationary phase culture was centrifuged at 3000g for 5 minutes and the supernatant was discarded. The cell pellet was transferred to a glass vial in 20µl sterile $H_2O$ to which 20µl 100% ethanol was added. This solution was then vortexed to mix followed by the addition of 200µl 6% Chelex-100 (w/v). The solution was vortexed again and incubated at 98°C for 10 minutes. Following incubation the solution was centrifuged at 3000g for 10 minutes and 1µl of the supernatant was used per PCR reaction. Cultures testing positive for the transgene (Figure 22) were taken forward for FAMEs profiling and growth curve analysis.

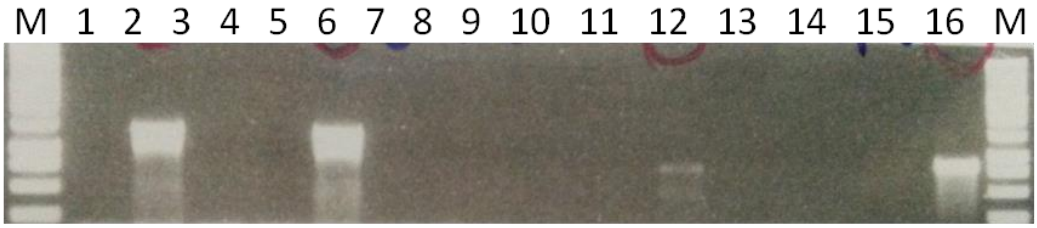



**Figure 22:** Colony PCR results confirming the presence of the transgene in lanes circled in red. Lanes 2 – 7 are from OtD6GFP cultures, lanes 9 – 16 FragElo6 cultures and lanes 1 and 8 are wild type negative controls.

The optimised colony PCR method developed for *T. pseudonana* proved to be reliable and efficient, saving considerable time. A further advantage is that it could also be applicable for high-throughput screening of cultures.

### 3.3.2 Growth curve analysis of transgenic and wild type *Thalassiosira pseudonana*

The first step in evaluating the overexpression lines in comparison to wild type *T. pseudonana* was to carry out a growth curve analysis. This was to assess if the overexpression of genes involved in omega-3 biosynthesis had any effect on growth rate, and also to determine when the exponential and stationary phases of growth occurred. Cell number per millilitre of culture was determined using a cell counter. All cultures were started at 5 x $10^5$ cells per ml (150ml culture in a 250ml flask) and incubated under constant illumination (100 µmol photons $m^{-2}$ $s^{-1}$) and temperature (20°C). Cells were counted every 24 hours for 20 days using a cell counter (Nexcelom Bioscience, Lawrence, MA). As can be seen in Figure 23, the growth curves follow a similar trend to that of wild type cells, so the overexpression of the transgenes does not appear to affect the growth characteristics of *T. pseudonana*.

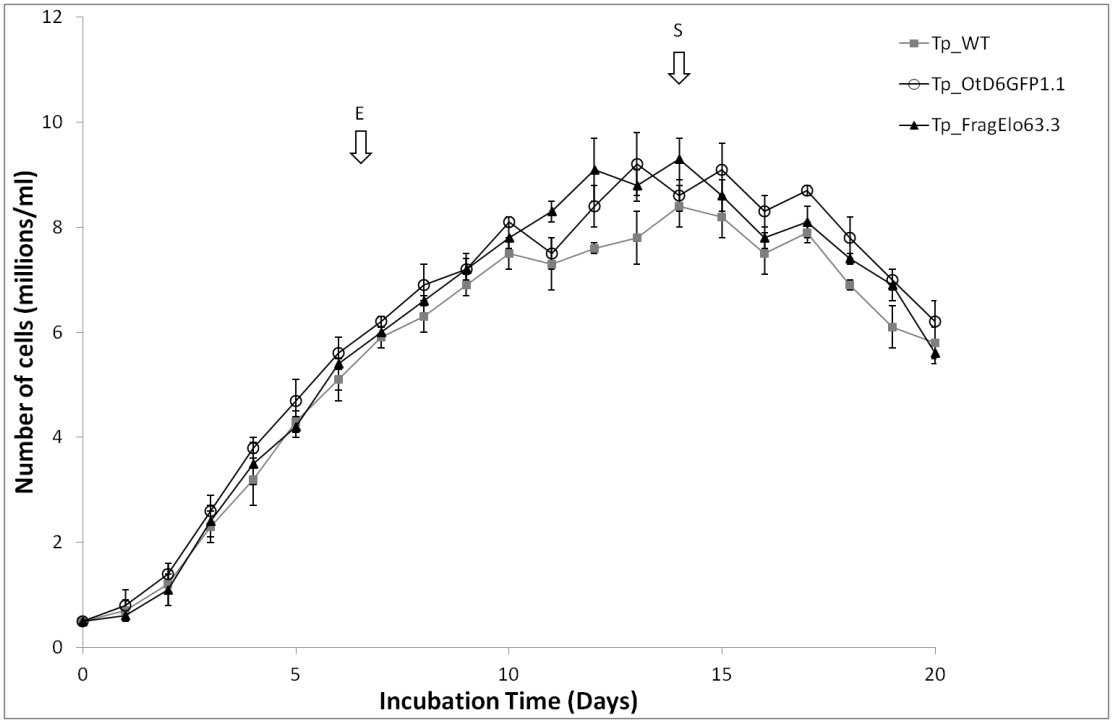



**Figure 23:** Growth curves for Tp_WT, Tp_OtD6GFP and Tp_FragElo6 cultures, from the mean of three biological replicates including error bars for standard deviation. The exponential (E) and stationary (S) phases are indicated by arrows. (NB – Tp_OtElo5 is not included after pilot studies revealed no change in fatty acid composition compared to WT. This line was therefore assessed separately in comparison only to WT).

The growth curves were used to approximate the number of days the cultures needed to be incubated before being sampled at a mid-stationary point for FAMEs analysis. Cultures were harvested at day 14 and 15mg (wet weight) samples were methylated for FAMEs analysis.

### 3.3.3 Fatty acid profiling of transgenic *T. pseudonana* overexpressing heterologous genes involved in omega-3 PUFA biosynthesis

Transgenic *T. pseudonana* cultures overexpressing heterologous genes involved in omega-3 PUFA biosynthesis were analysed using GC-FID and GC-MS to determine the fatty acid methyl ester (FAME) composition. Samples were harvested from mid-stationary phase cultures, as determined by standard growth curves for wild type and each of the transgenic lines. Preliminary FAMEs profiling revealed promising results for transgenic lines, with variations observed in fatty acid composition, when compared to wild type *T. pseudonana*. Cultures observed as having the most consistent differences were taken forward for fatty acid profiling over a number of technical and biological repeats, and also over a longer time period.

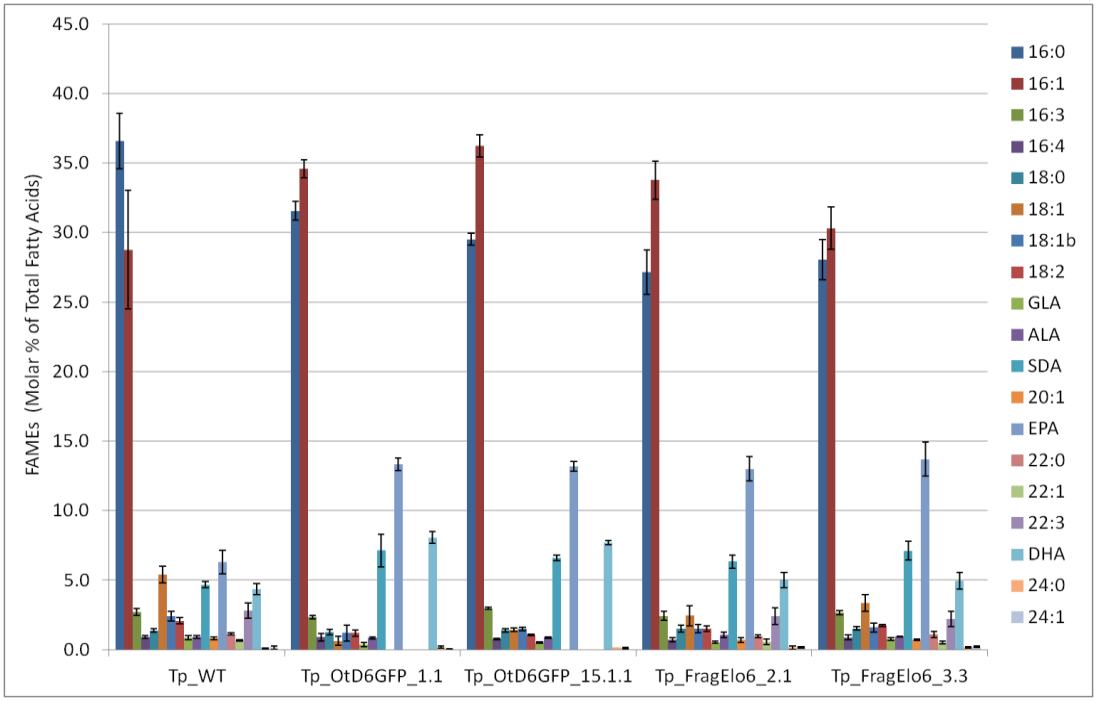



**Figure 24:** Fatty acid profiles of Tp_WT and transgenic *T. pseudonana* in December 2013. Values are the mean of nine replicates (3 technical x 3 biological repeats). Error bars represent standard deviation.

| Fatty Acid | Tp_WT | Tp_OtD6GFP_1.1 | Tp_OtD6GFP_15.1.1 | Tp_FragElo6_2.1 | Tp_FragElo6_3.3 |
|---|---|---|---|---|---|
| 16:0 | 36.6 ± 2.0 | 31.6 ± 0.7 | 29.5 ± 0.4 | 27.2 ± 1.6 | 28.1 ± 1.4 |
| 16:1 | 28.8 ± 4.3 | 34.6 ± 0.6 | 36.2 ± 0.8 | 33.8 ± 1.4 | 30.3 ± 1.5 |
| 16:3 | 2.7 ± 0.2 | 2.3 ± 0.1 | 3.0 ± 0.1 | 2.4 ± 0.3 | 2.7 ± 0.2 |
| 16:4 | 0.9 ± 0.1 | 0.9 ± 0.3 | 0.8 ± 0.0 | 0.7 ± 0.1 | 0.9 ± 0.2 |
| 18:0 | 1.4 ± 0.1 | 1.3 ± 0.2 | 1.4 ± 0.1 | 1.5 ± 0.3 | 1.5 ± 0.1 |
| 18:1 | 5.4 ± 0.6 | 0.6 ± 0.3 | 1.4 ± 0.1 | 2.4 ± 0.7 | 3.4 ± 0.6 |
| 18:1b | 2.4 ± 0.3 | 1.2 ± 0.6 | 1.5 ± 0.1 | 1.5 ± 0.3 | 1.6 ± 0.3 |
| 18:2 | 2.1 ± 0.2 | 1.2 ± 0.2 | 1.0 ± 0.1 | 1.5 ± 0.2 | 1.7 ± 0.1 |
| GLA | 0.9 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.0 | 0.5 ± 0.1 | 0.7 ± 0.1 |
| ALA | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.9 ± 0.0 | 1.0 ± 0.2 | 0.9 ± 0.0 |
| SDA | 4.7 ± 0.2 | 7.1 ± 1.2 | 6.6 ± 0.2 | 6.3 ± 0.5 | 7.1 ± 0.7 |
| 20:1 | 0.78 ± 0.1 | ND | ND | 0.7 ± 0.2 | 0.7 ± 0.1 |
| EPA | 6.3 ± 0.9 | 13.3 ± 0.4 | 13.2 ± 0.4 | 13.0 ± 0.9 | 13.7 ± 1.2 |
| 22:0 | 1.1 ± 0.1 | ND | ND | 1.0 ± 0.1 | 1.1 ± 0.2 |
| 22:1 | 0.7 ± 0.1 | ND | ND | 0.6 ± 0.2 | 0.5 ± 0.1 |
| 22:3 | 2.8 ± 0.5 | ND | ND | 2.4 ± 0.6 | 2.2 ± 0.6 |
| DHA | 4.3 ± 0.4 | 8.1 ± 0.4 | 7.7 ± 0.1 | 5.0 ± 0.5 | 4.9 ± 0.6 |
| 24:0 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.0 |
| 24:1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.0 |

**Table 6:** Fatty acid compositions of Tp_WT and transgenic *T. pseudonana* in December 2013 shown in Figure 24. Values are the mean of nine replicates ± standard deviation. ND – not detected.

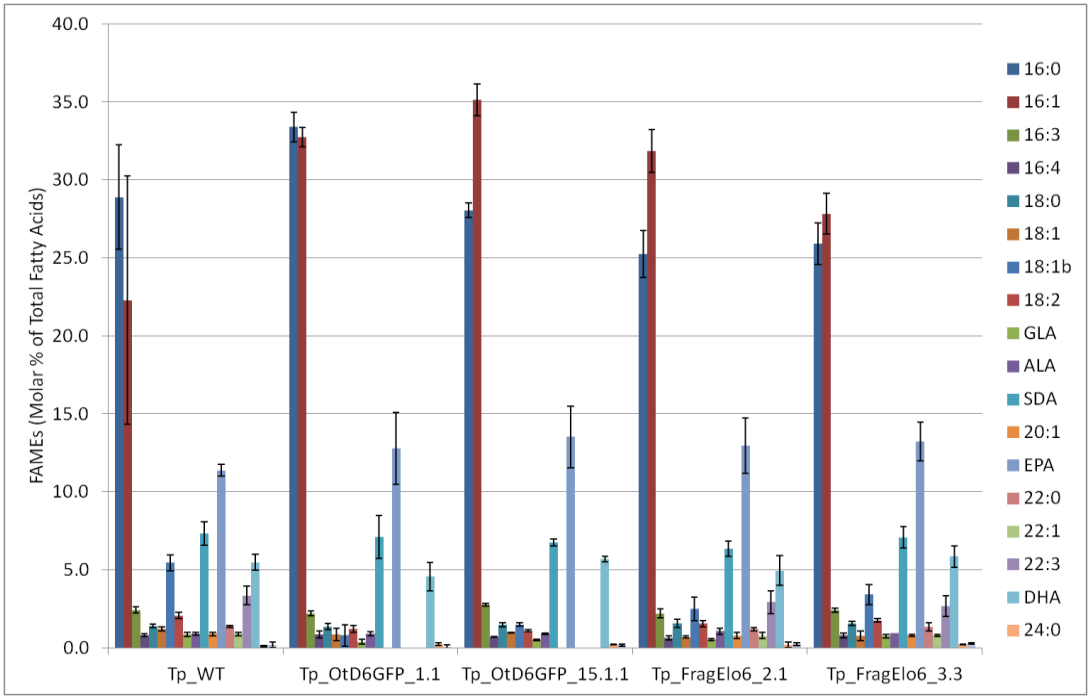



**Figure 25:** Fatty acid profiles of Tp_WT and transgenic *T. pseudonana* in March 2014. Values are the mean of nine replicates (3 technical x 3 biological repeats). Error bars represent standard deviation.

| Fatty Acid | Tp_WT | Tp_OtD6GFP_1.1 | Tp_OtD6GFP_15.1.1 | Tp_FragElo6_2.1 | Tp_FragElo6_3.3 |
|---|---|---|---|---|---|
| 16:0 | 28.9 ± 3.3 | 33.4 ± 0.9 | 28.0 ± 0.5 | 25.2 ± 1.5 | 25.9 ± 1.3 |
| 16:1 | 22.3 ± 8.0 | 32.7 ± 0.6 | 35.1 ± 1.0 | 31.8 ± 1.4 | 27.8 ± 1.3 |
| 16:3 | 2.4 ± 0.2 | 2.2 ± 0.1 | 2.8 ± 0.1 | 2.2 ± 0.3 | 2.4 ± 0.1 |
| 16:4 | 0.8 ± 0.1 | 0.8 ± 0.2 | 0.7 ± 0.0 | 0.6 ± 0.1 | 0.8 ± 0.1 |
| 18:0 | 1.4 ± 0.1 | 1.3 ± 0.2 | 1.5 ± 0.1 | 1.6 ± 0.3 | 1.6 ± 0.1 |
| 18:1 | 1.2 ± 0.1 | 0.8 ± 0.4 | 1.0 ± 0.0 | 0.7 ± 0.1 | 0.8 ± 0.3 |
| 18:1b | 5.4 ± 0.5 | 0.8 ± 0.7 | 1.5 ± 0.1 | 2.5 ± 0.8 | 3.4 ± 0.6 |
| 18:2 | 2.1 ± 0.2 | 1.2 ± 0.2 | 1.1 ± 0.1 | 1.5 ± 0.2 | 1.7 ± 0.1 |
| GLA | 0.9 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.0 | 0.5 ± 0.1 | 0.7 ± 0.1 |
| ALA | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.0 | 1.1 ± 0.2 | 0.9 ± 0.0 |
| SDA | 7.3 ± 0.7 | 7.1 ± 1.4 | 6.8 ± 0.2 | 6.4 ± 0.5 | 7.1 ± 0.7 |
| 20:1 | 0.9 ± 0.1 | ND | ND | 0.8 ± 0.2 | 0.8 ± 0.1 |
| EPA | 11.4 ± 0.4 | 12.8 ± 2.3 | 13.5 ± 2.0 | 13.0 ± 1.8 | 13.2 ± 1.2 |
| 22:0 | 1.4 ± 0.1 | ND | ND | 1.2 ± 0.1 | 1.3 ± 0.3 |
| 22:1 | 0.9 ± 0.1 | ND | ND | 0.8 ± 0.2 | 0.8 ± 0.1 |
| 22:3 | 3.3 ± 0.6 | ND | ND | 2.9 ± 0.7 | 2.7 ± 0.7 |
| DHA | 5.5 ± 0.5 | 4.6 ± 0.9 | 5.7 ± 0.2 | 5.0 ± 0.9 | 5.8 ± 0.7 |
| 24:0 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.2 | 0.2 ± 0.0 |
| 24:1 | 0.2 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.0 |

**Table 7:** Fatty acid compositions of Tp_WT and transgenic *T. pseudonana* in March 2014 shown in Figure 25. Values are the mean of nine replicates ± standard deviation. ND – not detected.

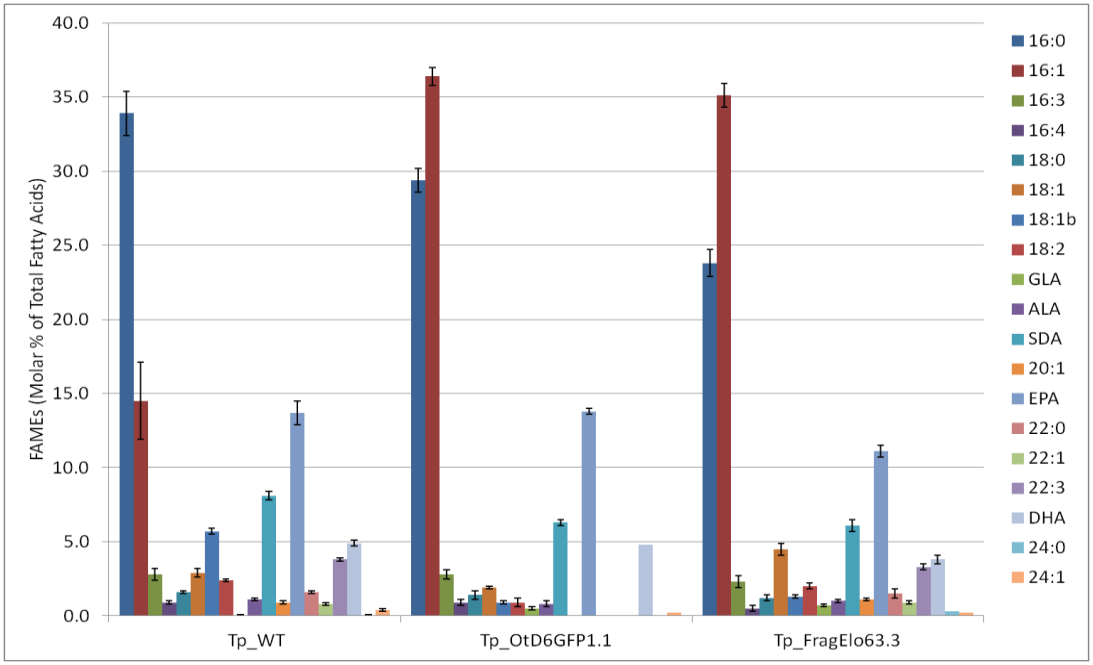



**Figure 26:** Fatty acid profiles of Tp_WT and transgenic T. pseudonana in May 2014. Values are the mean of nine replicates (3 technical x 3 biological repeats). Error bars represent standard deviation.

| Fatty Acid | Tp_WT | Tp_OtD6GFP1.1 | Tp_FragElo63.3 |
|---|---|---|---|
| 16:0 | 33.9 ± 1.5 | 29.4 ± 0.8 | 23.8 ± 0.9 |
| 16:1 | 14.5 ± 2.6 | 36.4 ± 0.6 | 35.1 ± 0.8 |
| 16:3 | 2.8 ± 0.4 | 2.8 ± 0.3 | 2.3 ± 0.4 |
| 16:4 | 0.9 ± 0.1 | 0.9 ± 0.2 | 0.5 ± 0.2 |
| 18:0 | 1.6 ± 0.1 | 1.4 ± 0.3 | 1.2 ± 0.2 |
| 18:1 | 2.9 ± 0.3 | 1.9 ± 0.1 | 4.5 ± 0.4 |
| 18:1b | 5.7 ± 0.2 | 0.9 ± 0.1 | 1.3 ± 0.1 |
| 18:2 | 2.4 ± 0.1 | 0.9 ± 0.3 | 2.0 ± 0.2 |
| GLA | 1 .0 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.1 |
| ALA | 1.1 ± 0.1 | 0.8 ± 0.2 | 1.0 ± 0.1 |
| SDA | 8.1 ± 0.3 | 6.3 ± 0.2 | 6.1 ± 0.4 |
| 20:1 | 0.9 ± 0.1 | ND | 1.1 ± 0.1 |
| EPA | 13.7 ± 0.8 | 13.8 ± 0.2 | 11.1 ± 0.4 |
| 22:0 | 1.6 ± 0.1 | ND | 1.5 ± 0.3 |
| 22:1 | 0.8 ± 0.1 | ND | 0.9 ± 0.1 |
| 22:3 | 3.8 ± 0.1 | ND | 3.3 ± 0.2 |
| DHA | 4.9 ± 0.2 | 4.8 ± 0.0 | 3.8 ± 0.3 |
| 24:0 | 0.0 ± 0.1 | ND | 0.3 ± 0.0 |
| 24:1 | 0.4 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.0 |

**Table 8:** Fatty acid compositions of Tp_WT and transgenic *T. pseudonana* in May 2014 shown in Figure 26. Values are the mean of nine replicates ± standard deviation. ND – not detected.

Despite successful transformation events and numerous positive transformants, none of the Tp_OtElo5 clones showed any variation in fatty acid composition when compared to wild type. These were therefore not included in the multiple biological and technical repeat experiments, but were monitored separately in comparison to wild type *T. pseudonana* over time. No significant variation, as determined by ANOVA, was observed between Tp_OtElo5 and wild type FAMEs profiles (not shown) over the course of this PhD.

Interestingly, over the two year period that FAMEs profiling was carried out, an almost seasonal cycle was observed in the fatty acid composition of both wild type and transgenic lines. Figure 24, Figure 25 and Figure 26 show the FAMEs profiles of Tp_WT versus Tp_OtD6GFP and Tp_FragElo6 cultures at different periods throughout 2013 – 2014 (December 2013, March 2014 and May 2014 respectively). These compositions are also shown in Table 6, Table 7 and Table 8 as molar % of total fatty acids (again, December 2013, March 2014 and May 2014 respectively). These results are also representative of similar profiles that were observed in January 2013, April 2013 and July 2013, correspondingly. The results are the mean of nine replicates (three technical x three biological repeats). ANOVA was used to demonstrate that no statistically significant difference was present between the three biological replicates.

The most abundant fatty acid in Tp_WT in all periods examined was 16:0, followed by 16:1 (36.6 – 28.9% and 28.8 – 14.5% of total fatty acids, respectively). Levels of EPA varied from 6.3% (December 2013), to 11.4% (March 2014) to 13.7% (May 2014), and DHA ranged from 4.3% (December 2013) to 5.5% (March 2014) to 4.9% (May 2014). SDA ranged from 4.7% (December 2013) to 8.1% (May 2014).

In all transgenic lines during all periods tested the most abundant fatty acid was 16:1, followed by 16:0. Tp_OtD6GFP lines varied in SDA and EPA from 6.3 – 7.1% and 12.8 – 13.8% respectively, but the most variation was observed in the levels of DHA, ranging from a high of 8.1% (December 2013) to 4.6% (March 2014). No intermediary fatty acids (20:1, 22:0, 22:1 and 22:3) were observed at any time in OtD6GFP lines, but they were detected in small amounts (<5%) in Tp_FragElo6 lines. The Tp_FragElo6 lines had a minimum DHA level of 3.8% (May 2014) and maximum of 5.8% (March 2014), and EPA levels ranged from 11.1% (May 2014) to 13.7% (December 2013).

PCR was carried out on all lines, including wild type, before FAMEs analysis, using primers for all transgenes using the colony PCR method described above. This was firstly to confirm the transgene was still present, and secondly to rule out any cross contamination between

cultures. In all cultures tested transgenes remained detectable for the duration of this PhD and no cross contamination occurred.

In order to examine the data more closely, levels of each fatty acid were compared separately for each line in December 2013, March 2014 and May 2014. Fatty acids with notable variation (as determined by student's T-Test), either between lines or within lines, are shown in Figure 27.

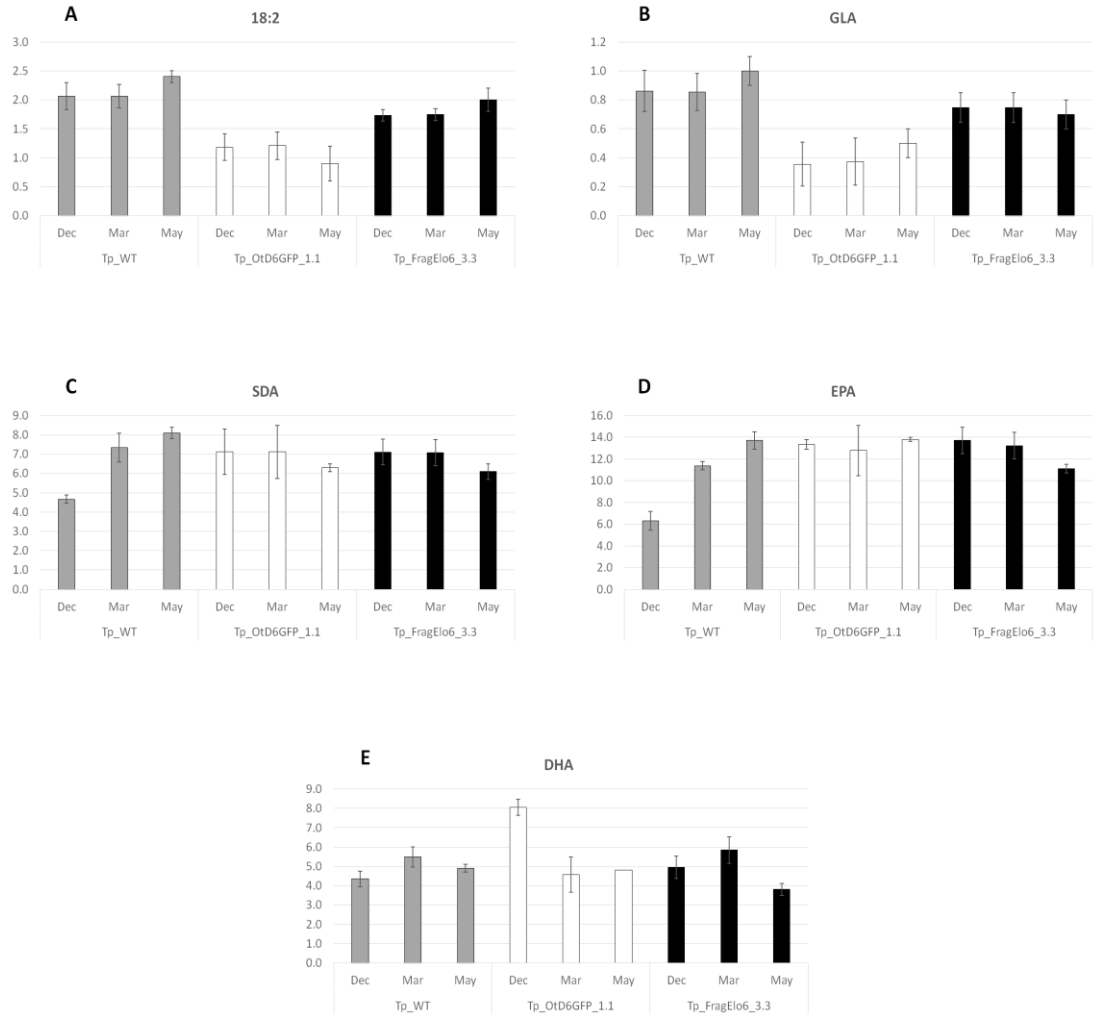



**Figure 27:** Comparison of the levels of 18:2 (A), GLA (B), SDA (C), EPA (D) and DHA (E) in and between Tp_WT, Tp_OtD6GFP and Tp_FragElo6 over time.

Firstly, it can be seen more clearly that in Tp_OtD6GFP and Tp_FragElo6 there has been an effect on the levels of substrate for the respective transgenic enzymes that have been overexpressed. Figure 27A shows that the level of 18:2 (substrate for Δ6-desaturase) is significantly lower (reduction range between 45.4%, $P<0.001$, and 62.6%, $P<0.01$, for December 2013 and May 2014 respectively) in Tp_OtD6GFP lines than in Tp_WT. A similar trend is also seen for GLA (substrate for Δ6-elongase) in the Tp_FragElo6 lines, with a reduction range between 19.4%, $P<0.05$, and 30.0%, $P<0.05$ (in December 2013 and May 2014, respectively) when compared to Tp_WT (Figure 27B), although this variation has a low statistical significance ($P<0.05$). These trends are stable over the time period tested and would suggest that the transgenic proteins are functional, desaturating or elongating their respective substrates. Interestingly, the level of GLA is also significantly reduced in Tp_OtD6GFP lines (reduction range between 61.0%, $P<0.001$, and 50.0%, $P<0.005$, for December 2013 and May 2014 respectively). This may be due to the increased desaturation of 18:2 resulting in an increased flux at this early stage of the omega-3 biosynthetic pathway, therefore causing the level of GLA to be reduced. However, it should be noted that the amount of GLA across all lines is in the range of 1.0% or lower, therefore the instrument sensitivity, or lack thereof, may have influenced the level of variation detected.

Perhaps the most interesting variation is observed when data for SDA and EPA are examined (Figure 27C and D). Significant variation is detected in the levels of these fatty acids in Tp_WT over time, with both increasing from December 2013 to March 2014, and from March 2014 to May 2014. This change appears to be almost seasonal (this observation is re-enforced when the FAMEs profile for wild type *T. pseudonana* from an independent experiment in Chapter 4 is taken into account, as it also follows this trend). A similar phenomenon has been reported previously (Pernet et al, 2003). However, in Pernet's and colleagues' study, this could likely be attributed to seasonal fluctuations in seawater composition since, although they conducted their experiment under controlled light, temperature and pH, they used natural filtered seawater to culture the microalgae.

A number of environmental factors can affect the lipid and fatty acid composition of microalgae; these include temperature (James et al, 1989; Thompson and Guo, 1992; Thompson, 1992), nutrient availability (Shifrin and Chisholm, 1981; Piorreck et al, 1984; Taguchi et al, 1987; Lombardy and Wangersky, 1991; Sukenik and Wahnon, 1991), light intensity (Shifrin and Chisholm, 1981; Sukenik and Wahnon, 1991) and culture growth phase (Piorreck and Pohl, 1984; Taguchi et al, 1987). The variability associated with these conditions

was mitigated in this PhD study by growing *T. pseudonana* cultures under constant conditions (temperature, light, artificial seawater with pre-determined nutrient concentrations and pH).

This leads to the supposition that other factors may also have an effect on fatty acid composition, and potentially on other cell features. Extensive research has been carried out on microalgal circadian and seasonal rhythms (Johnson et al, 1991; Jacobshagen and Johnson, 1994; Fábregas et al, 2002; Golden and Canales, 2003; Depauw et al, 2012; Braun et al, 2014; see Noordally and Millar (2014) and Raible and Falciatore (2014) for extensive reviews). The regulation and level of gene expression in microalgae is closely tied in with these rhythms. In *T. pseudonana* this has been demonstrated by the identification of large scale anticipatory circadian mechanisms affecting the regulation of gene expression (Ashworth et al, 2013); for example, during diurnal growth, the transcriptional states of genes involved in fatty acid biosynthesis are significantly upregulated at dawn, following 12 hours of darkness, and significantly downregulated following 12 hours of daylight. The up/downregulation of such genes precedes the onset of light or dark conditions (Ashworth et al, 2013). Microalgae also synchronise cell division in a circadian manner, ensuring harmonisation with their photosynthetic activity (Harding et al, 1984; Goto and Johnson, 1995; Lüning et al, 1997). Diurnal cycles are also important for determining seasonal rhythms in microalgae (Pfeuty et al, 2012), but this could not explain the results observed during this PhD, since cultures were grown under constant light and temperature, with no diel cycles present. Non-transcriptional clocks have been identified in *Ostreococcus* sp. (Bouget et al, 2014), but no such studies on *T. pseudonana* could be found. Importantly, long-running rhythms of varying periodicity (e.g. lunar or tidal rhythms) have been observed in marine species (Tessmar-Raible et al, 2011; Raible and Falciatore 2014), which contrast with model terrestrial species which only display overt circadian rhythms, and occasionally seasonal rhythms. This implies that multiple clock-like mechanisms exist in marine species, including diatoms, whose anticipatory nature allow them to maintain periodicity, even if external environmental cues are missing or misleading.

A recent paper on the green alga *Dunaliella viridis* (Bozhkov et al, 2014) found that growth under constant "ideal" conditions (24 hour illumination, constant nutrients and temperature) did not eliminate the rhythmic nature of biomass accumulation, and pronounced variability was observed in nucleic acid, protein and TAG composition throughout the year of study. These annual dynamics had a periodicity of approximately 4 months and coincided with solar activity and changes in electromagnetic radiation – something that cannot be controlled in a standard lab. Electromagnetic radiation could potentially act as a trigger for the periodicity

observed. Further investigation would be required to determine if a similar effect is occurring in *T. pseudonana*.

If regulation of gene expression is occurring as the result of a clock mechanism, this may be responsible for the periodic increase in SDA and EPA content in Tp_WT, and could explain why there is a concurrent decrease in SDA, EPA and DHA during May 2014 (Figure 27C, D and E), and increase of intermediates (Table 8) in the Tp_FragElo6 cultures. If the increase in SDA and EPA in Tp_WT is caused by the downregulation of the endogenous Δ6-elongase-encoding gene, it could be expected that there would be an accumulation of SDA. The regulatory elements may not be functional on the transgenic *FragElo6*, so some Δ6-elongase activity would still be occurring. Therefore, the decrease in EPA and DHA in Tp_FragElo6 may be the result of decreased native Δ6-elongase activity.

Another noteworthy intra-line variation to highlight is that for DHA content in Tp_OtD6GFP (Figure 27E). DHA was significantly higher than Tp_WT in December 2013 (increase of 84.5%, $P<0.001$), but this was not the case for March or May 2014, with the level DHA falling markedly when compared to December 2013 (reduction of 43.3%, $P<0.001$, and 40.1%, $P<0.001$ for December 2013 vs. March 2014 and December 2013 vs. May 2014 respectively). This variation was initially attributed to silencing of the transgene over time, however, as highlighted above, the activity of the transgenic Δ6-desaturase appears to remain stable over the time period tested, and DHA levels were also found to be high in preliminary experiments, so this hypothesis can be rejected. No obvious explanation for this periodic sharp decrease in DHA Tp_OtD6GFP could be identified.

### 3.3.4 Acyl-CoA pool profiling of transgenic *T. pseudonana* overexpressing OtD6GFP and FragElo6

The composition of the acyl-CoA pools were profiled to provide further information about omega-3 PUFA biosynthesis in wild type and transgenic *T. pseudonana*. Acyl-CoA profiling was undertaken at the same time as FAMEs analysis in December 2013. Figure 28 and Figure 29 provide the same acyl-CoA data in two formats to allow the comparison of the overall acyl-CoA composition and profile of each line, as well as the distribution of specific acyl-CoA pools between the lines. Exact values for these data are reported in Table 9.

Acyl-CoA profiling revealed that the most abundant pool in Tp_WT was 20:5-CoA, followed by 16:0-CoA and 16:1-CoA, which are in line with results previously reported for *T.*

*pseudonana* (Tonon et al, 2005a). These are also the most abundant fatty acids observed in the FAMEs profile (Figure 24); however, this relationship is not stoichiometric. In all transgenic lines 16:0-CoA was the most abundant pool, followed by 20:5-CoA, then 16:1-CoA for Tp_OtD6GFP and 24:0-CoA for Tp_FragElo6. The levels of 16:0-CoA in all transgenic lines are higher than that of the same CoA pool in Tp_WT. The reasons for this are unclear; however, one explanation is that it could be the result of increased beta-oxidation resulting in higher levels of 16:0-CoA in these lines.

It is worth noting that the third most abundant pool in Tp_FragElo6 is 24:0-CoA, when in FAMEs analysis this fatty acid was only detected between 0.1 – 0.2% of total fatty acids. No obvious explanation could be identified for such a phenomenon. It is likely that the reason for this could be identified with further analysis of total lipids in this line.

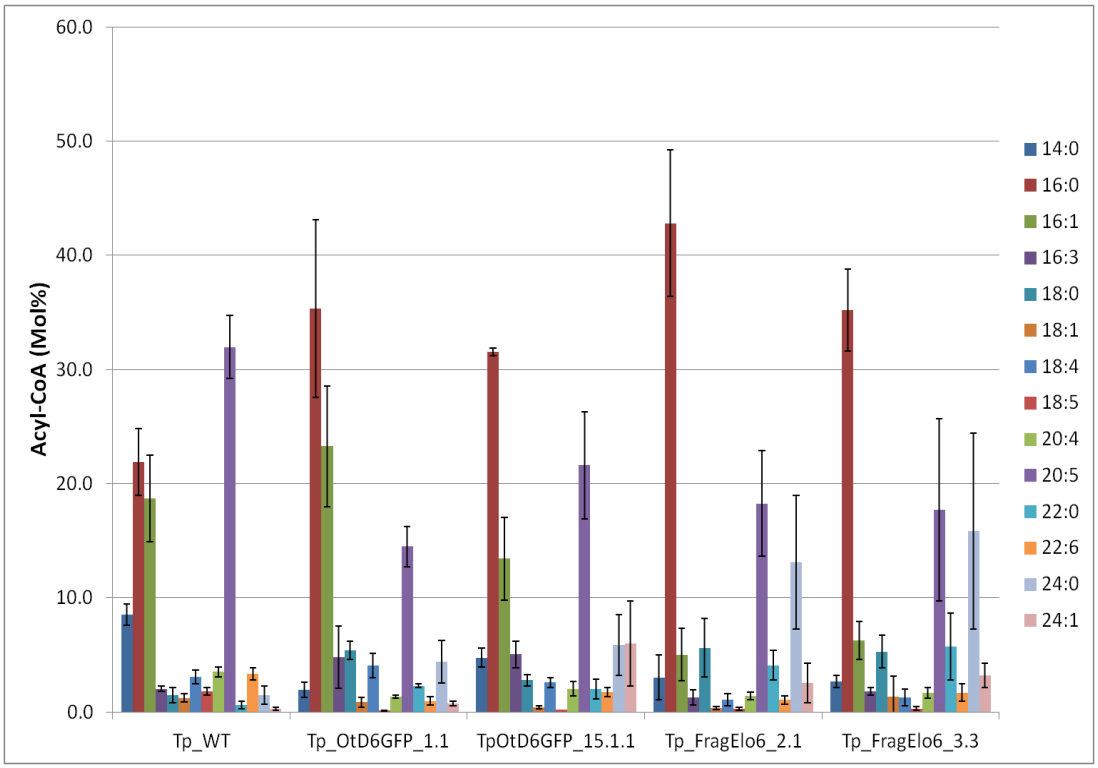



**Figure 28:** Acyl-CoA composition (mol%) of Tp_WT and transgenic *T. pseudonana* lines from samples harvested during the mid-stationary phase of growth, as determined by standard growth curves. Values are the mean of three technical replicates. Error bars represent the standard deviation.

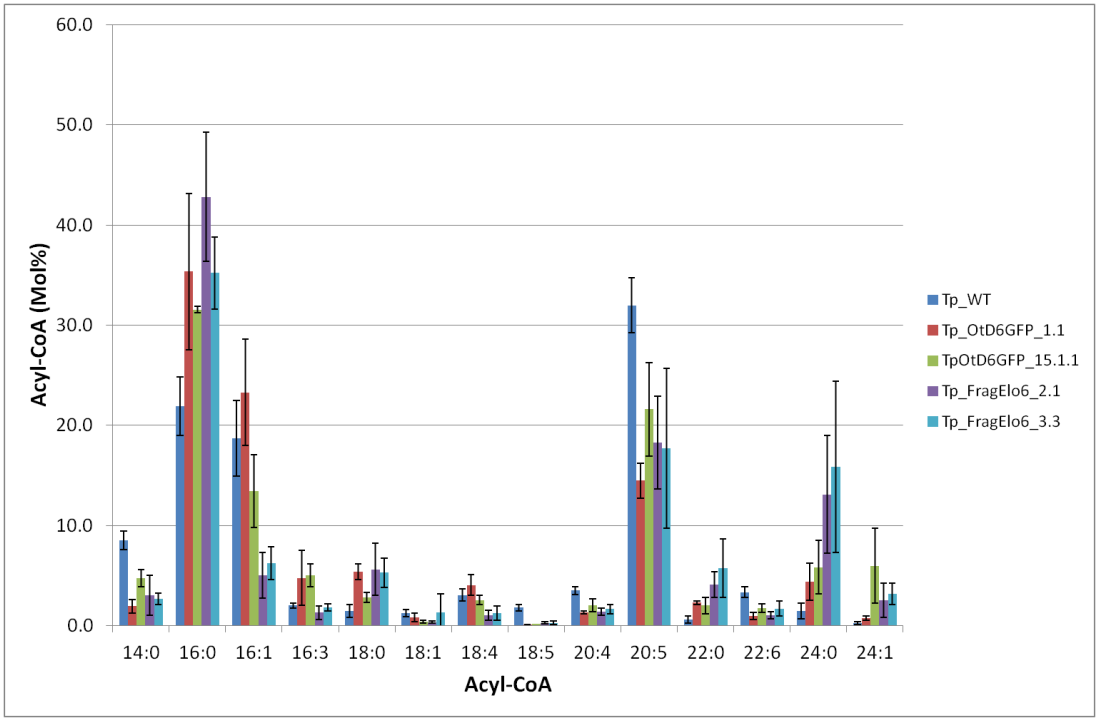



**Figure 29:** Comparison of the distribution of acyl-CoA pools (mol%) of Tp_WT and transgenic *T. pseudonana* lines from samples harvested during the mid-stationary phase of growth, as determined by standard growth curves. Values are the mean of three technical replicates. Error bars represent the standard deviation.

| Acyl CoA | Tp_WT | Tp_OtD6GFP_1.1 | TpOtD6GFP_15.1.1 | Tp_FragElo6_2.1 | Tp_FragElo6_3.3 |
|---|---|---|---|---|---|
| 14:0 | 8.5 ± 0.9 | 1.9 ± 0.7 | 4.8 ± 0.9 | 3.0 ± 2.0 | 2.7 ± 0.6 |
| 16:0 | 21.9 ± 2.9 | 35.3 ± 7.8 | 31.5 ± 0.3 | 42.8 ± 6.4 | 35.2 ± 3.6 |
| 16:1 | 18.7 ± 3.8 | 23.3 ± 5.3 | 13.4 ± 3.6 | 5.0 ± 2.3 | 6.3 ± 1.6 |
| 16:3 | 2.0 ± 0.3 | 4.8 ± 2.7 | 5.1 ± 1.2 | 1.3 ± 0.7 | 1.8 ± 0.4 |
| 18:0 | 1.5 ± 0.7 | 5.4 ± 0.8 | 2.8 ± 0.5 | 5.6 ± 2.6 | 5.3 ± 1.5 |
| 18:1 | 1.2 ± 0.4 | 0.8 ± 0.4 | 0.4 ± 0.2 | 0.4 ± 0.1 | 1.4 ± 1.8 |
| 18:4 | 3.1 ± 0.6 | 4.1 ± 1.0 | 2.6 ± 0.4 | 1.1 ± 0.5 | 1.3 ± 0.7 |
| 18:5 | 1.8 ± 0.3 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.2 |
| 20:4 | 3.5 ± 0.4 | 1.3 ± 0.1 | 2.0 ± 0.6 | 1.4 ± 0.4 | 1.7 ± 0.5 |
| 20:5 | 32.0 ± 2.7 | 14.5 ± 1.8 | 21.6 ± 4.7 | 18.3 ±4.6 | 17.7 ± 8.0 |
| 22:0 | 0.6 ± 0.3 | 2.3 ± 0.2 | 2.0 ± 0.8 | 4.1 ± 1.3 | 5.7 ± 2.9 |
| 22:6 | 3.3 ± 0.5 | 1.0 ± 0.4 | 1.7 ± 0.4 | 1.1 ± 0.4 | 1.7 ± 0.7 |
| 24:0 | 1.5 ± 0.8 | 4.4 ± 1.8 | 5.8 ± 2.7 | 13.1 ± 5.9 | 15.9 ± 8.6 |
| 24:1 | 0.3 ± 0.2 | 0.7 ± 0.2 | 6.0 ± 3.7 | 2.5 ± 1.7 | 3.2 ± 1.1 |

**Table 9:** Acyl-CoA composition (mol%) of Tp_WT and transgenic *T. pseudonana* lines from samples harvested during the mid-stationary phase of growth, as determined by standard growth curves. Values are the mean of three technical replicates ± standard deviation.

Another important observation worth highlighting is the presence of 18:5-CoA in Tp_WT. Although this unusual (n-1) fatty acid has been previously detected in some marine species (Parsons et al, 1961; Volkman et al, 1981), a literature search that was conducted couldn't find any other reports of 18:5 in *T. pseudonana*. 18:5 is derived from the Δ15-desaturation of 16:3 (n-4). It may be that FAMEs analysis wasn't sensitive enough to detect 18:5 as it also doesn't appear in any of the FAMEs profiles for this line obtained during this PhD.

Interestingly, the amount of 20:5-CoA and 22:6-CoA is less in transgenic lines compared to wild type, despite being higher in the FAMEs composition (Figure 24 and Table 6). These results suggest that one alternative approach for engineering wild type *T. pseudonana* could be the overexpression of a DGAT gene (native or heterologous) for the acylation of these activated forms of EPA and DHA into neutral lipids, for their integration into TAG. This approach may result in the increased accumulation of EPA and DHA in TAG species. Furthermore, analysis of TAG species in the Tp_OtD6GFP and Tp_FragElo6 transgenic lines may reveal that the incorporation of EPA and DHA into TAG is higher than in Tp_WT, providing a possible explanation for this phenomenon. However, the definitive experiments to determine this remain to be carried out. If this is the case it would be interesting to determine the effect of co-expression of genes encoding DGAT activity alongside those expressing either Δ6-desaturase or Δ6-elongase activity.

Further consideration should also be given to the role of LACS (long-chain acyl-CoA synthetases) in the accumulation of fatty acids in TAGs in *T. pseudonana*. It is postulated that ACS (acyl-CoA synthetases) are important in determining the size and composition of the acyl-CoA pool (Tonon et al, 2005b), however, it is not clear how this influences fatty acid accumulation, nor are the mechanisms that regulate the acyl-CoA pool fully understood. It may be that LACS play a role in the regulation of PUFA acyl-CoA production, and could therefore be one important factor controlling the flux and availability of PUFA acyl-CoA substrates for incorporation into TAG.

### 3.3.5 Imaging studies of transgenic *T. pseudonana* overexpressing OtD6GFP and FragElo6

#### 3.3.5.1 Localisation of transgenic GFP-tagged Δ6-desaturase in Tp_OtD6GFP

Confocal microscopy was used to determine the localisation of the transgenic GFP-tagged Δ6-desaturase in Tp_OtD6GFP lines (Figure 30). It was also used as a second screening method alongside colony PCR to identify positive OtD6GFP transformants.

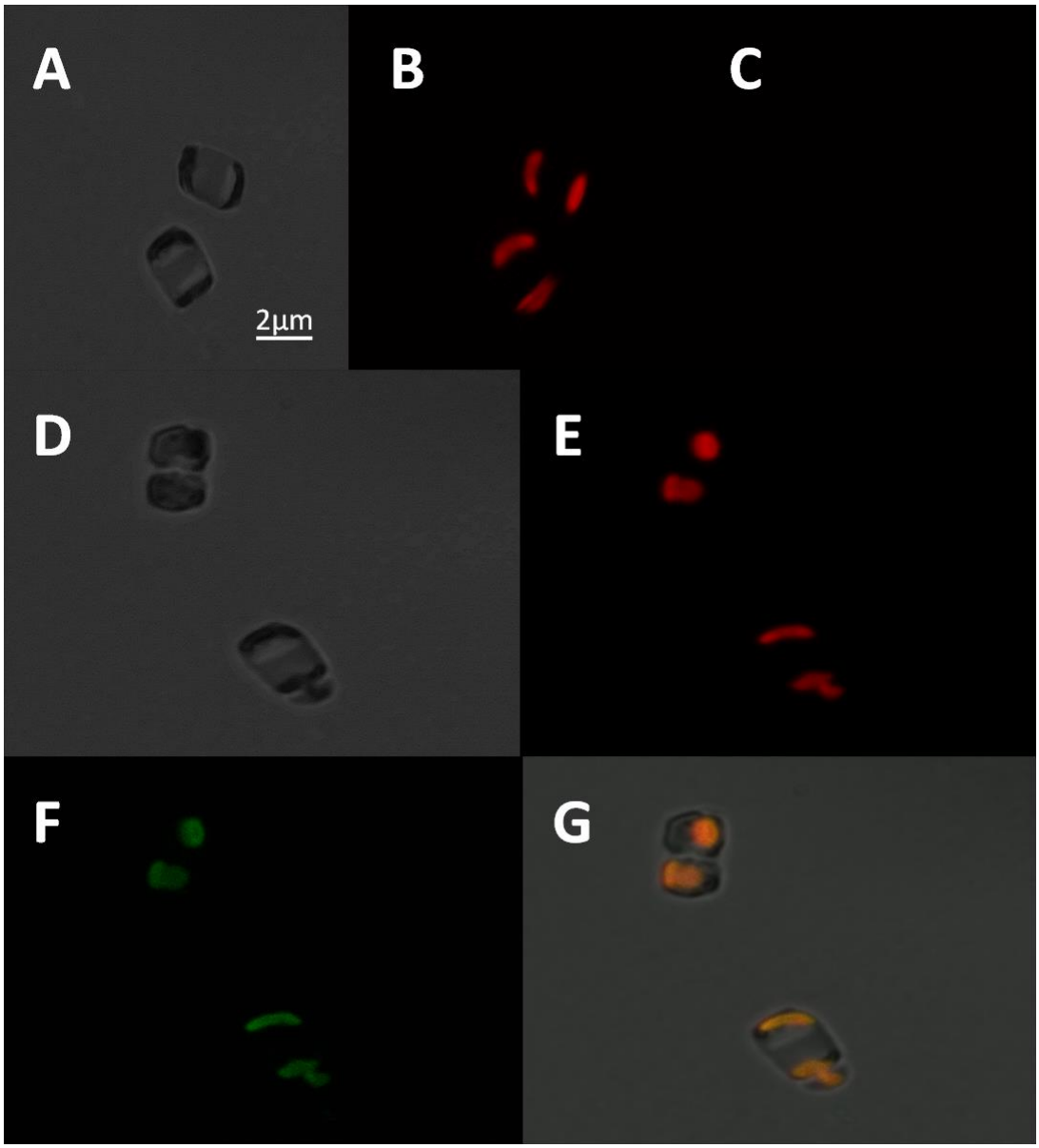



**Figure 30:** Confocal microscopy images of stationary phase Tp_WT (A – brightfield image; B – red indicates chlorophyll autofluorescence; C – No fluorescence is observed when exposed under a GFP filter) and Tp_OtD6GFP cells (D – brightfield image; E – chlorophyll autofluorescence; F – green indicates GFP, and thus the localisation of the transgenic GFP-fused protein; G – merge of images D, E and F).

Figure 30 shows Tp_WT (A, B and C) versus Tp_OtD6GFP cells (D, E, F and G). These images show that GFP fluorescence is only detected for Tp_OtD6GFP cells, indicating the presence of the transgenic Δ6-desaturase. The merge shows that transgenic Δ6-desaturase is also localised to the plastids, indicated by the overlap of green fluorescence for GFP with the red fluorescence for chlorophyll. These results are encouraging because they show that transgenic Δ6-desaturase is being targeted to the correct cellular compartment in order for it to carry out its function.

#### 3.3.5.2 Phenotypic effects of transgenic genes encoding Δ6-desaturase and Δ6-elongase in Tp_OtD6GFP and Tp_FragElo6

Confocal microscopy studies were also carried out to determine if the overexpression of genes encoding Δ6-desaturase and Δ6-elongase had any phenotypic effects on *T. pseudonana* cells (Figure 31). BODIPY was used to stain lipid droplets so that these could be visualised and compared between lines. All imaging studies were undertaken on live cells from the mid-stationary phase of growth.

Tp_WT cells exhibit a normal phenotype (top row of Figure 31) with chloroplasts located at either end of the cell (in opposite frustules). BODIPY staining shows the presence of one major lipid droplet with one or two much smaller ones, which may be plastoglobules. The large lipid droplets appear to be associated with the chloroplast, suggesting they are formed from the thylakoid membrane.

There also appears to be some co-localisation of lipid droplets with the chloroplast in Tp_OtD6GFP (middle row of Figure 31). Interestingly, given the pattern of chlorophyll fluorescence (shown in red) it seems that the chloroplasts have a markedly altered morphology compared to those in Tp_WT. The chloroplasts in Tp_OTD6GFP are stretched and flattened, appearing to be thinner and more lengthened than those observed in Tp_OtD6. The Tp_OtD6GFP cells also seem to be slightly longer and narrower than Tp_WT cells, however further imaging studies measuring numerous cells would be required to determine if this is a significant phenotype.

It could be concluded that the presence of a greater number of larger lipid droplets signifies that there is more TAG in these cells; however, a full total lipid analysis would be required to quantify not only the amount of TAG, but also the distribution of fatty acids across the lipid classes.

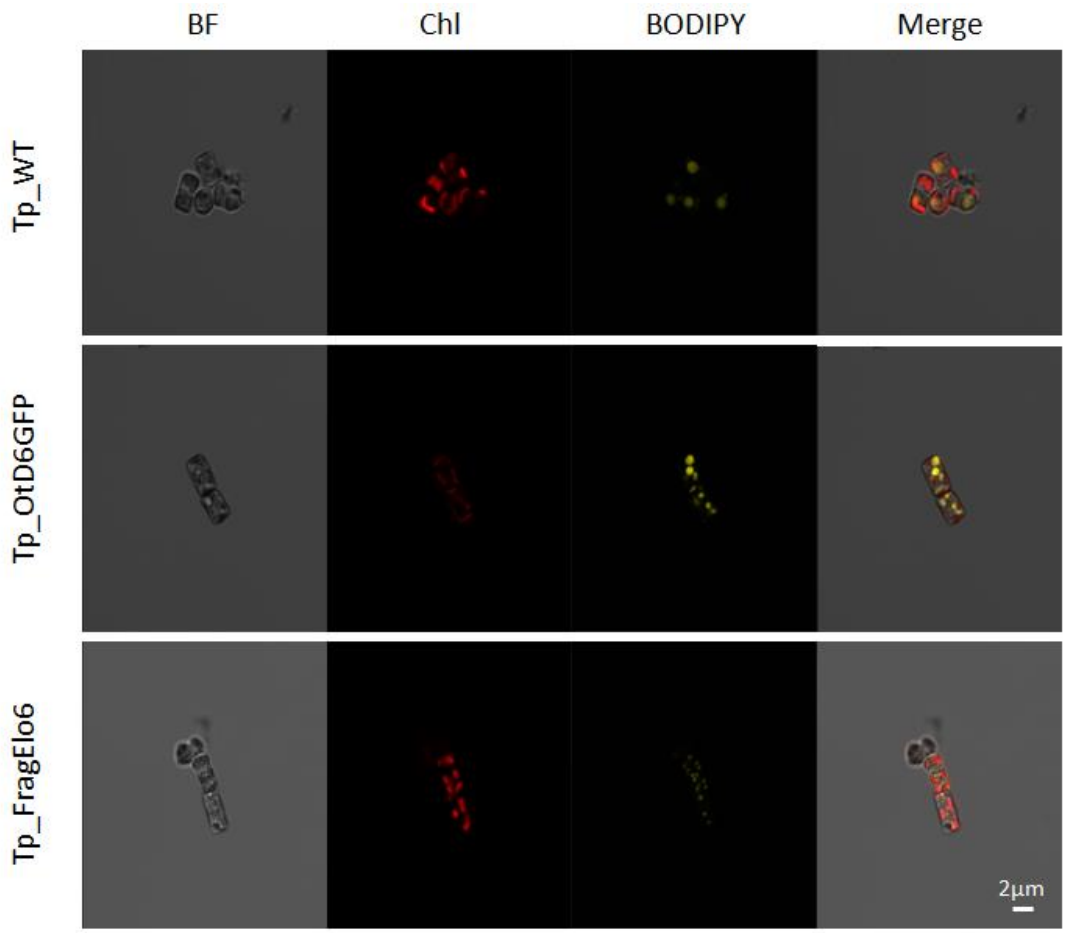



**Figure 31:** Confocal microscopy images of stationary phase Tp_WT, Tp_OtD6GFP and Tp_FragElo6 comparing brightfield (BF), chlorophyll (Chl, in red) and BODIPY (yellow) fluorescence.

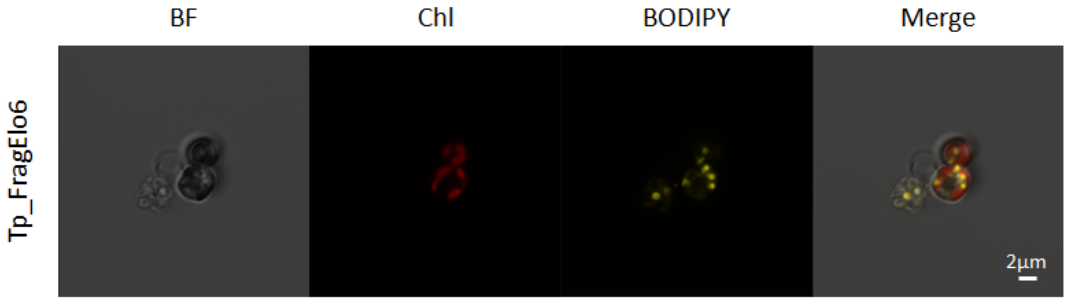



**Figure 32:** Confocal microscopy images of stationary phase Tp_FragElo6 comparing brightfield (BF), chlorophyll (Chl, in red) and BODIPY (yellow) fluorescence. View from the top of cells showing the thylakoid stacks (red chlorophyll fluorescence).

In contrast to the lipid droplet distribution in Tp_WT and Tp_OtD6GFP cells, in Tp_FragElo6 cells they are more numerous (bottom row of Figure 31), but a lot smaller and dispersed throughout the cell. It is not clear if all of these are plastoglobules or if they are just smaller lipid droplets formed from the thylakoid membrane. The cells also appear to be noticeably elongated, and almost stretched, compared to Tp_WT cells. Again, an altered morphology of the chloroplasts can be seen; they are more numerous and appear to be somewhat fractionated. Figure 32 shows a view from the top of a group of Tp_FragElo6 cells, with visible thylakoid stacks and associated lipid droplets.

The images obtained from confocal microscopy studies demonstrate a clear visual difference between Tp_WT, Tp_OTD6GFP and Tp_FragElo6 cells. The precise reasons for the diverse chloroplast and lipid droplet morphologies are unclear. A total lipid analysis may shed light on the source for this variation; for example, a dramatic change in the composition of membrane lipid classes may result in changes to membranous organelles, such as chloroplasts and lipid droplets. One reason for this could be the increase in longer chain fatty acids in Tp_OtD6GFP and Tp_FragElo6, particularly in the acyl-CoA pools (Table 9). Studies in plants (Millar et al, 1998) have found that the accumulation of very long chain fatty acids (VLC-FAs) results in altered thylakoid membrane structure, as a result of altered membrane lipid composition. VLC-FAs are normally found in sphingolipids, which are important structural membrane lipids. It has been suggested that thylakoid membrane lipids containing VLC-FAs may mimic the structural role of sphingolipids, causing curvature of the membranes. Further investigation would be required to determine if this is the source of altered chloroplast morphology in transgenic *T. pseudonana* cells.

## 3.4 CONCLUSIONS

If the results for December 2013 were taken in isolation one could conclude that the overexpression of genes encoding Δ6-desaturase and Δ6-elongase activities had a positive enhancement on the levels of SDA, EPA and DHA in *T. pseudonana*. This was also the trend that was observed in preliminary experiments the previous year. Encouragingly, overexpression of OtD6 and FragElo6 didn't lead to any detrimental effects on the growth rate. However, due to the variation observed over time, particularly with regards to Tp_WT, no firm conclusions can be made other than overexpression of genes encoding Δ6-desaturase and Δ6-elongase activities in *T. pseudonana* does have an impact on the FAMEs and acyl-CoA profiles, as well as producing a varying phenotype when compared to wild type cells. This

may not be the optimal technique for enhancing EPA and DHA production in *T. pseudonana*, since improved EPA and DHA synthesis is not enhanced greatly nor is any change observed stable over time. The level of enhancement is skewed by the significant variations observed in the wild type. Further investigation is required to determine the source of this variation in wild type *T. pseudonana* cells, which may result in further need for a different approach to enhancing EPA and DHA production and accumulation in this species.

Lipid metabolism in *T. pseudonana* is likely to be a complex process, influenced by many factors, as supported by this study. Knockout or knock-down investigations, coupled with full metabolomics studies would be required to learn more about lipid metabolism in *T. pseudonana*, to fully understand all factors at play and allow for the rational metabolic engineering of this microalgal species. Furthermore, the design of multigene expression vectors and/or new engineering techniques will permit the manipulation of multiple steps in multiple pathways, potentially evading the accumulation of unusual fatty acids or byproducts which may result in unwanted physiological or morphological changes.

# Chapter 4

# Gene Silencing Of Omega-3 Biosynthetic Activity In *Thalassiosira pseudonana*

## 4.1 ABSTRACT

One of the major drawbacks of studying diatom biology is the lack of tools to generate targeted knock-down and knockout mutants. Recent advances in the development of molecular tools to manipulate diatoms have enabled the possibility to trigger gene silencing using constructs containing inverted repeat sequences of targeted genes. This approach has previously been successful in the diatom *Phaeodactylum tricornutum*. As part of this PhD, the same methodology was applied to *Thalassiosira pseudonana* to silence native genes involved in omega-3 biosynthesis, namely those encoding Δ9-desaturase, Δ5-desaturase, Δ4-desaturase and Δ6-elongase activities. Within the scope of this study, no effect on overall polyunsaturated fatty acid production was observed, with no significant difference observed between FAMEs profiles of wild type versus modified lines. These results could be due to this technique not having been successful or inactivation of the silencing construct by *T. pseudonana*. Despite no variations observed in the FAMEs profiles, the growth rate of the mutant line targeted for the knock-down of Δ9-desaturase activity was negatively impacted, with concurrent observations of altered cell shape and size. This may be the result of a toxic effect of the inverted repeat construct or silencing of off-target genes vital for normal cell function, resulting in deleterious effects to cell growth characteristics. Alternatively, this could indicate functional redundancy between additional (sequence-unrelated) Δ9-desaturases, which allowed for the homeostasis of Δ9-desaturation, but generated a state of "haploinsufficiency" which impacted on the growth of the cells. Given the results obtained in this study it is suggested that future functional genomics investigations utilise alternative techniques to uncover more information about the roles of the above genes in endogenous lipid metabolism in *T. pseudonana*.

## 4.2 INTRODUCTION

Analysis of whole genome sequences from the diatoms *Thalassiosira pseudonana* (Armbrust et al, 2004; Montsant et al, 2007) and *Phaeodactylum tricornutum* (Bowler et al, 2008) reveal the presence of interesting metabolic features derived from their complex evolutionary history (Armbrust et al, 2004), which includes the uptake of a vast number of genes through horizontal gene transfer (Bowler et al, 2008). Such features include those typical of plants, such as photosynthetic pathways, and those normally found in animal cells, for example a urea cycle (Ambrust et al, 2004) (further information on diatom biology can be found in Chapter 1: Introduction). Comparative analyses of the genomes of these two diatoms revealed major differences in their genome structure and a significant number of genes of unknown function (Bowler et al, 2008). These diatom specific genes are yet to be fully characterised and represent a major challenge for the comprehensive understanding of diatom biology.

Over the past few years an increasing number of molecular tools and techniques have been developed to study and manipulate diatoms (Montsant et al, 2005; Siaut et al, 2007; Kroth, 2007; Mock et al, 2008; Maheswari et al, 2009). One major limitation had been the lack of tools to generate knockout and knock-down mutants, using either forward or reverse genetic approaches. No homologous recombination events have been observed in diatoms (Falciatore et al, 1999), therefore the use of homologous recombination for targeted gene disruption is unlikely to be successful. Furthermore, the generation of loss-of-function mutants via an insertional mutagenesis approach is difficult in diploid organisms lacking a sexual cycle, such as *P. tricornutum* (De Riso et al, 2009).

Recent work by De Riso and colleagues successfully utilised constructs containing inverted repeat and antisense sequences for targeted gene silencing (De Riso et al, 2009). RNA-mediated interference (RNAi) can occur through a number of different processes which result in RNA silencing (Baulcombe, 2004; Brodersen and Voinnet, 2006; Chapman and Carrington, 2007; Hutvagner and Simard; 2008). Double-stranded RNA precursors (dsRNA) can generate RNA molecules between approximately 20 – 30 nucleotides which can regulate gene expression and are involved in these RNA silencing processes, significantly altering genome function by affecting transcription, translation, chromatin structure and RNA processing and stability (Carthew and Sontheimer, 2009; Voinnet, 2009). The manipulation of these regulatory processes mediated by RNAi has become a powerful tool for the study of functional genomics in a number of organisms, although exogenously triggered RNA silencing

methods remain the least understood of these techniques (Brodersen and Voinnet, 2006). The most common method used historically involved the expression of genes or gene fragments in an antisense orientation via transformation constructs to alter target gene expression (van der Krol et al, 1988; Nellen and Lichtenstein, 1993). Following research leading to the discovery that dsRNA was the ultimate trigger for gene silencing, new methods were developed, enhancing the gene silencing process via the simultaneous expression of sense and antisense constructs, or the use of invert repeat constructs to directly trigger the production of high levels of dsRNA (Hope, 2001; Dykxhoorn et al, 2003; Montgomery, 2004; Lamitina, 2006). Work continues in both prokaryotes and eukaryotes to develop a stable and efficient high-throughput gene silencing method (Watson et al, 2005; Schroda, 2006; Li et al, 2007; Ramadan et al, 2007).

For the purposes of this study a gene silencing method previously proven successful in the diatom *P. tricornutum* (De Riso et al, 2009) was used for the targeted knock-down of endogenous genes involved in omega-3 PUFA biosynthesis in the diatom *T. pseudonana*. Using transformation constructs containing inverted repeat sequences to targeted genes, namely the native Δ9-desaturase (Accession number: XM_002288140), Δ5-desaturase (Accession number: XM_002288806.1), Δ4-desaturase (Accession number: AY817156.1) and a Δ6-elongase (Accession number: AY591337.1), experiments were carried out to determine how silencing these genes would affect lipid metabolism in *T. pseudonana*. These genes were selected based on their roles in the biosynthesis of omega-3 PUFAs (Figure 7, Chapter 2). Δ5-desaturase and Δ4-desaturase are the final desaturation steps for the synthesis of EPA and DHA respectively (Armbrust et al, 2004; Tonon et al, 2005a). It can therefore be expected that a reduction in their activity could result in the reduced production of these fatty acids. The Δ6-elongase is responsible for the elongation of SDA to ETA (Meyer et al, 2004), which is then desaturated by Δ5-desaturase to EPA, as already described. The rational behind targeting this gene was to observe any effects on downstream EPA and DHA accumulation. Δ9-desaturase is responsible for the desaturation of palmitic acid (16:0) to palmitoleic acid (16:1). This target was chosen to investigate any impact on the downstream omega-6 and omega-3 pathways resulting from a decreased conversion of 16:0 to 16:1. Although no obvious knock-down effect was observed on the overall FAMEs profiles of the mutant lines, the line targeted for the knock-down of Δ9-desaturase activity exhibited a markedly reduced growth rate, with cultures rapidly deteriorating and cells dying.

## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Development of Gene Silencing Constructs and Transformation in *T. pseudonana*

To investigate the effects of gene silencing on the fatty acid profile of *T. pseudonana*, four transformation constructs were assembled containing inverted repeat sequences (De Riso et al, 2009) for target genes involved in PUFA biosynthesis and co-transformed into wild type *T. pseudonana* with a plasmid conferring resistance to the antibiotic nourseothricin. The genomic sequences of the four target native genes encoding Δ9-desaturase, Δ5-desaturase, Δ4-desaturase and Δ6-elongase activities were used to develop primers for inverted repeat sequences (*Des9*, *Des5*, *Des4* and *Elo6*, respectively). These inverted repeat sequences were inserted as *EcoRV-KpnI* fragments into the pTpFCP-GFP plasmid under the control of the constitutive FCP promoter (Figure 33). Each inserted fragment was designed to include a STOP codon at the 3'-end to prevent read-through and expression of GFP. This was done to eliminate any potential interference by the expression of GFP in the gene silencing process.

Following repeated unsuccessful transformation events, viable clones were eventually obtained for all four target genes. Once colonies on plates were scaled up to 150ml cultures, through transfer to liquid ESAW medium, a sample was taken and used to confirm the presence of the inverted repeat sequence fragment by PCR amplification of extracted DNA with specific primers for each fragment (Figure 34). Cells containing inverted repeat sequence fragments were found for each target gene and taken forward for subsequent FAMEs and growth curve analysis. These cultures will be referred to as Tp_Des9-, Tp_Des5-, Tp_Des4- and Tp_Elo6-. Cultures were kept in a controlled environment growth room under constant light (100µmol photons $m^{-2}$ $s^{-1}$) and temperature (20°C).

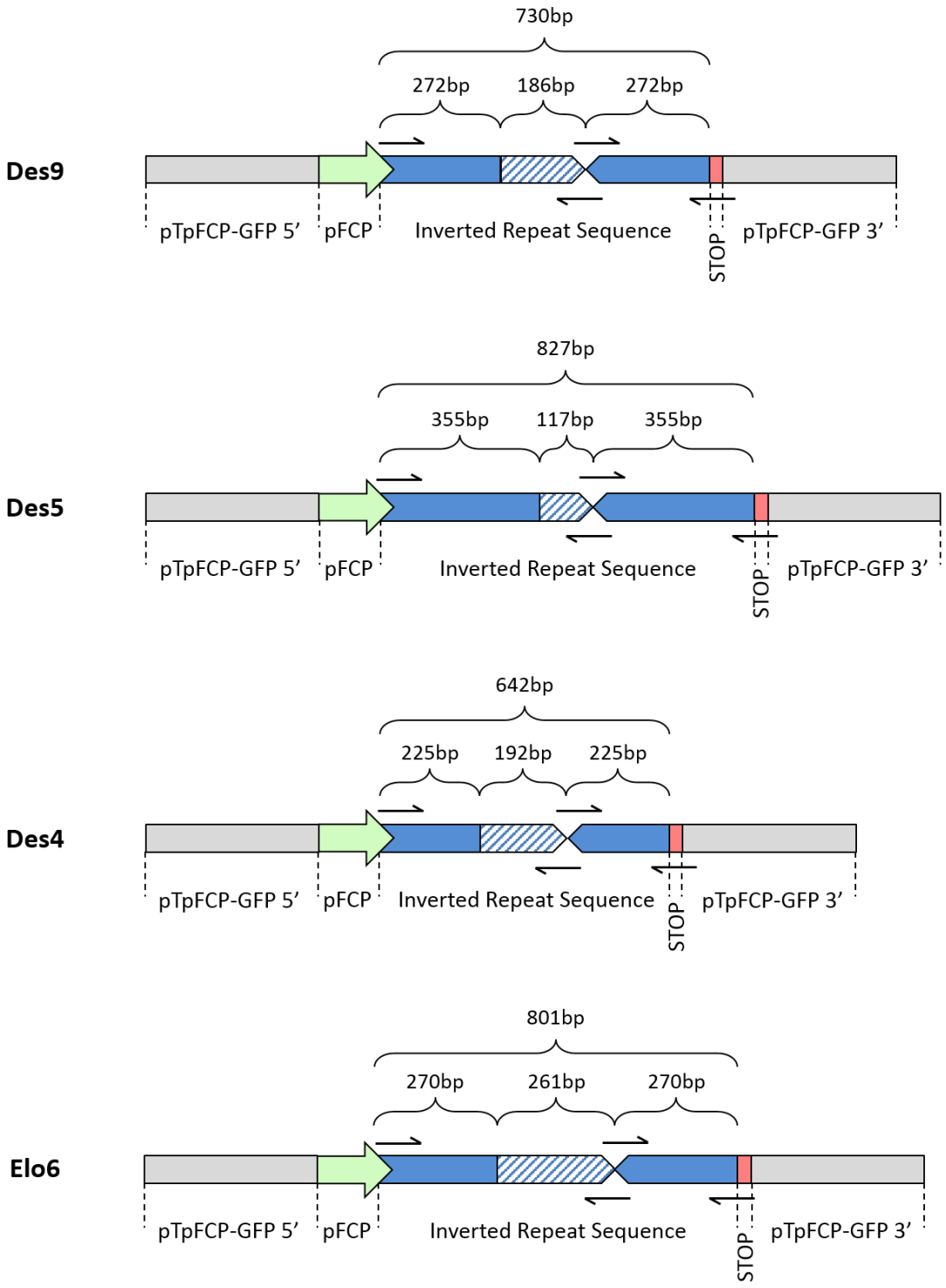



**Figure 33:** Schematic map of inverted repeat constructs. Inverted repeat sequences for target native genes were inserted as *EcoRV-KpnI* fragments under the control of the constitutive FCP promoter (pFCP). The self-complementary regions are shown in blue and the non-complimentary spacer region is shaded with diagonal lines. The spacer region is included to stabilise the hairpin loop structure. The STOP codon to prevent read-through and expression of GFP is shown in red. Sizes are shown for the whole fragment and complementary and non-complementary regions.

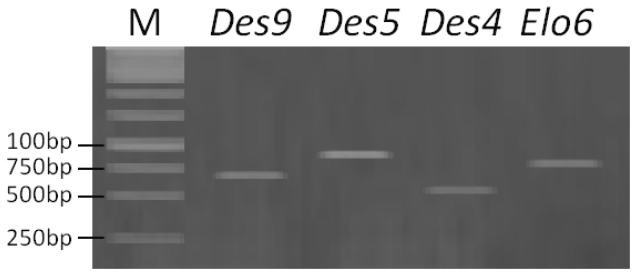



**Figure 34:** PCR amplifications performed on extracted DNA from *T. pseudonana* to confirm the presence of fragments containing inverted repeat sequences for gene silencing of native Δ9-desaturase (*Des9*), Δ5-desaturase (*Des5*), Δ4-desaturase (*Des4*) and Δ6-elongase (*Elo6*). The marker 1kb DNA ladder is also shown (M).

### 4.3.2 Effect of Targeted Gene Silencing on the Fatty Acid Profile of *T. pseudonana*

The main aim of this study was to determine the effect of silencing genes involved in PUFA biosynthesis on the fatty acid profile of each of the *T. pseudonana* cultures. Fatty acid profiling was carried out on mid-stationary phase culture samples, as determined by reference growth curves. Samples were harvested and 15mg wet weight was methylated and FAMEs were analysed using GC-FID and GC-MS. Due to the reduced growth rate of Tp_Des9- cultures (see 4.3.3) a wet weight sample of 15mg was unable to be obtained, so samples with wet weights between 8-10mg were used.

FAMEs profiling revealed that there was no significant difference between Tp_WT and any of the lines containing gene silencing constructs (Figure 33 and Table 10). Signal intensities from GC-FID appeared lower for Tp_Des9- (not shown), however, this could be the result of methylating samples with a lower wet weight. Further studies would be required to determine whether the total overall fatty acid content remains the same across these lines. But with no variation observed in the relative distribution of fatty acids it was decided not to pursue further lipidomic analyses of these lines.

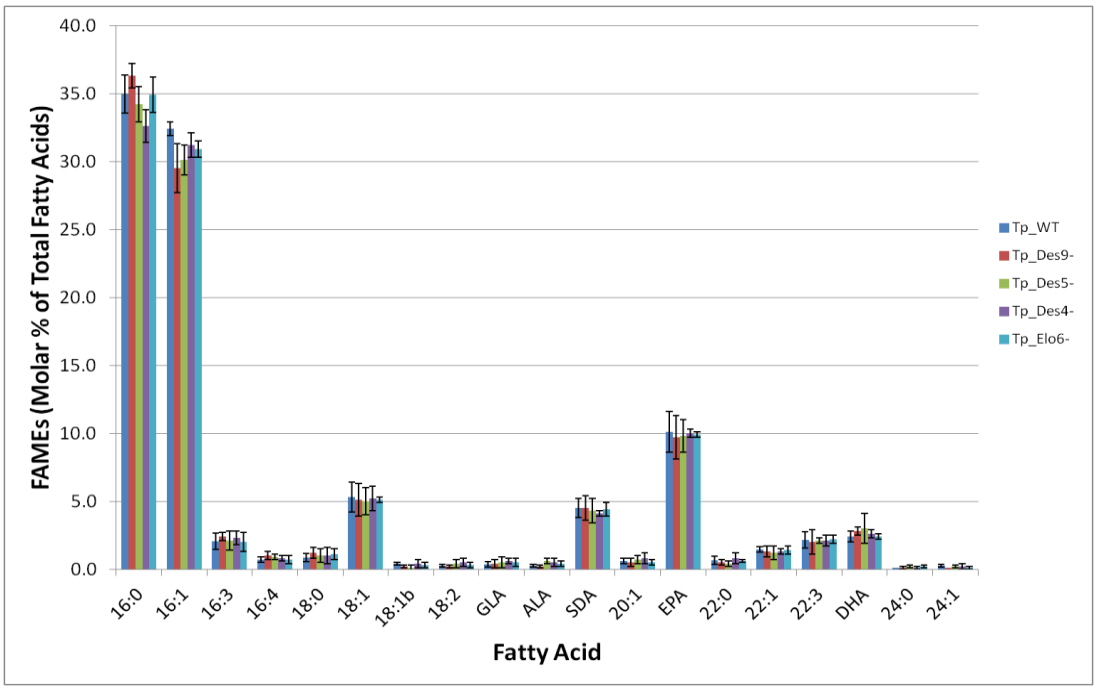



**Figure 35:** Comparison of distribution of fatty acids (mol%) of Tp_WT, Tp_Des9-, Tp_Des5-, Tp_Des4- and Tp_Elo6- lines from mid-stationary phase of growth. Values are the mean of three technical replicates. Error bars represent the standard deviation.

| Fatty Acid | Tp_WT | Tp_Des9- | Tp_Des5- | Tp_Des4- | Tp_Elo6- |
|---|---|---|---|---|---|
| 16:0 | 35.0 ± 1.4 | 36.3 ± 0.9 | 34.2 ± 1.3 | 32.6 ± 1.2 | 34.9 ± 1.3 |
| 16:1 | 32.4 ± 0.5 | 29.5 ± 1.8 | 30.1 ± 1.1 | 31.2 ± 0.9 | 30.9 ± 0.6 |
| 16:3 | 2.1 ± 0.6 | 2.4 ± 0.3 | 2.1 ± 0.7 | 2.3 ± 0.5 | 2.0 ± 0.7 |
| 16:4 | 0.7 ± 0.2 | 1.0 ± 0.3 | 0.9 ± 0.2 | 0.8 ± 0.2 | 0.7 ± 0.3 |
| 18:0 | 0.9 ± 0.3 | 1.2 ± 0.4 | 1.0 ± 0.5 | 1.0 ± 0.6 | 1.1 ± 0.4 |
| 18:1 | 5.3 ± 1.1 | 5.1 ± 1.2 | 5.0 ± 1.0 | 5.2 ± 0.9 | 5.1 ± 0.2 |
| 18:1b | 0.4 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.2 | 0.4 ± 0.3 | 0.3 ± 0.2 |
| 18:2 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.3 | 0.5 ± 0.3 | 0.3 ± 0.2 |
| GLA | 0.4 ± 0.2 | 0.4 ± 0.3 | 0.5 ± 0.4 | 0.6 ± 0.2 | 0.5 ± 0.3 |
| ALA | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.6 ± 0.2 | 0.5 ± 0.3 | 0.4 ± 0.2 |
| SDA | 4.5 ± 0.7 | 4.5 ± 0.9 | 4.3 ± 0.9 | 4.1 ± 0.2 | 4.4 ± 0.5 |
| 20:1 | 0.6 ± 0.2 | 0.5 ± 0.3 | 0.7 ± 0.3 | 0.8 ± 0.4 | 0.5 ± 0.2 |
| EPA | 10.1 ± 1.5 | 9.7 ± 1.6 | 9.8 ± 1.2 | 10.0 ± 0.3 | 9.9 ± 0.2 |
| 22:0 | 0.7 ± 0.3 | 0.5 ± 0.2 | 0.4 ± 0.2 | 0.8 ± 0.4 | 0.6 ± 0.1 |
| 22:1 | 1.5 ± 0.2 | 1.3 ± 0.4 | 1.2 ± 0.5 | 1.3 ± 0.2 | 1.4 ± 0.3 |
| 22:3 | 2.2 ± 0.6 | 2.0 ± 0.9 | 2.1 ± 0.2 | 2.1 ± 0.4 | 2.2 ± 0.3 |
| DHA | 2.4 ± 0.4 | 2.8 ± 0.3 | 3.0 ± 1.1 | 2.6 ± 0.3 | 2.4 ± 0.2 |
| 24:0 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 |
| 24:1 | 0.3 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.1 |

**Table 10:** Fatty acid compositions of Tp_WT and transgenic *T. pseudonana* in May 2014 shown in Figure 26. Values are the mean of nine replicates ± standard deviation. ND – not detected.

The FAMEs results would suggest that gene silencing did not work in this study. This could be due to either the constructs not generating any or high enough levels of dsRNA, or that a different region of the gene of interest needs to be targeted. Or, indeed, that the silencing construct was itself silenced by native *T. pseudonana* cellular machinery. A detailed *in silico* analysis of *P. tricornutum* and *T. pseudonana* genomes for known components of the RNAi pathway (De Riso et al, 2009) found that molecular factors involved in RNA silencing in other eukaryotes are only poorly conserved in diatoms. Therefore evolutionarily distantly related proteins may fulfil these functions in these diatoms. This may also mean that similar silencing approaches used in other model organisms cannot be directly translated to diatoms.

### 4.3.3 Effect of Targeted Gene Silencing on the Growth Rate of *T. pseudonana*

Perhaps the most interesting results from this study came after analysis of growth curves for Tp_Des9-, Tp_Des5-, Tp_Des4- and Tp_Elo6-. All cultures were started at 5 x $10^5$ cells per ml (150ml culture in a 250ml flask) and incubated under constant illumination (100 µmol photons $m^{-2}$ $s^{-1}$) and temperature (20°C). Cells were counted every 24 hours for 20 days using a cell counter (Nexcelom Bioscience, Lawrence, MA). While no significant difference was observed in the growth characteristics of Tp_Des5-, Tp_Des4- and Tp_Elo6- compared to Tp_WT, Tp_Des9- exhibited markedly reduced growth rate and reduced cell number (Figure 36). Tp_Des9- cultures were also difficult to retrieve once the cell number fell below 5 x $10^5$ cells per ml.

While cell counts were undertaken, notes were made on cell shape and size. Tp_Des9- cells were noted to be noticeably smaller than wild type cells and also appeared to be more square-shaped than the rectangular Tp_WT. No variations in cell appearance were seen for Tp_Des5-, Tp_Des4- and Tp_Elo6- cultures.

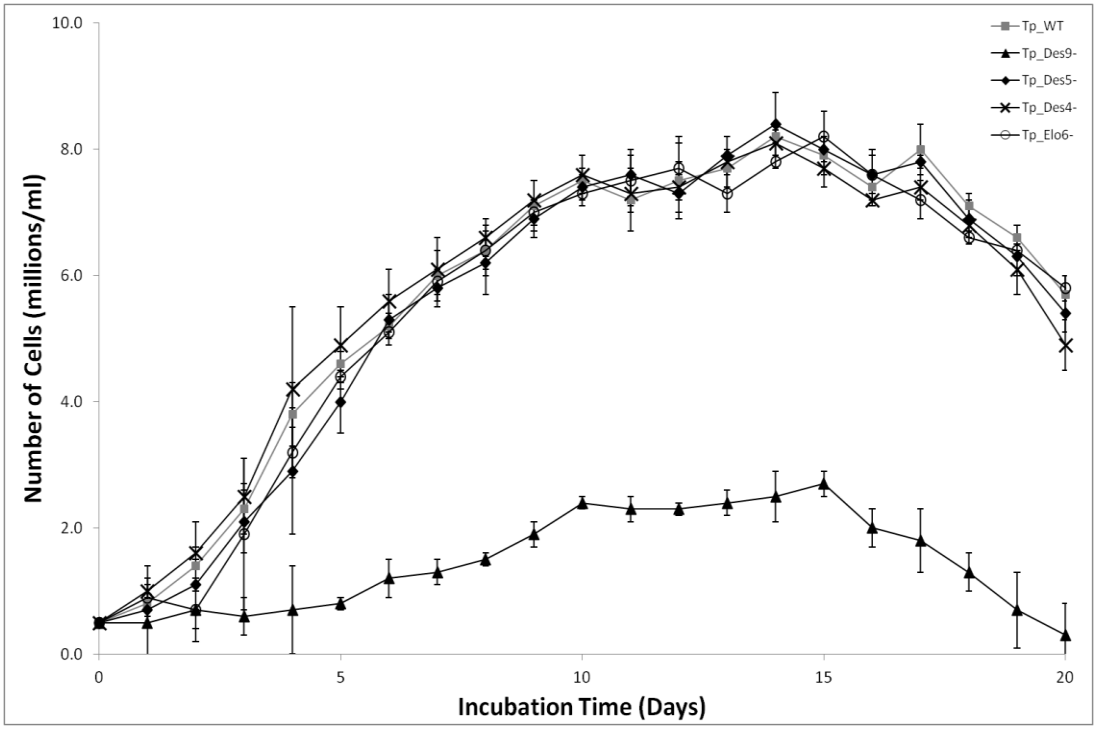



**Figure 36:** Growth curves for Tp_WT, Tp_Des9-, Tp_Des5-, Tp_Des4- and Tp_Elo6- cultures, from the mean of three biological replicates including error bars for standard deviation. Samples were harvested on Day 14 for FAMEs analysis.

It may be that the inverted repeat fragment targeting *Des9* was somehow toxic to the *T. pseudonana* cells, resulting in reduced cell number and growth rate. Alternatively, the inverted repeat sequence used may have resulted in the silencing of a non-target native gene that is vital to the cell, causing deleterious effects to cell health.

The effects on the growth characteristics of Tp_Des9- were not investigated further as this was not the purpose of this study. It is likely that any future work on silencing native *T. pseudonana* genes encoding Δ9-desaturase, Δ5-desaturase, Δ4-desaturase and Δ6-elongase, or other omega-3 PUFA biosynthetic activities, will be undertaken using an alternative method (see conclusions).

A consistent barrier encountered during this study was the very limited number of transformants obtained after each round of transformation, if any at all. No more than two or three positive transformants were obtained each time for Tp_Des5-, Tp_Des4- and Tp_Elo6-, and not more than one per transformation for Tp_Des9-. However, it cannot be concluded that the inverted repeat constructs were toxic to the cell since low transformation

efficiency has been a problem encountered throughout the duration of this PhD, including in transformations carried out for overexpression studies (Chapter 3).

A possible alternative explanation, and perhaps the most logical, for the lack of variation in FAMEs and the growth phenotype observed in the Tp_Des9- line is that there was an effect on the target Δ9-desaturase and its activity, but this was masked by increased activity of another, functionally redundant, protein that escaped sequence-based silencing. Such a protein is likely to be derived from a structurally unrelated gene to that encoding Des9. In order to investigate such a theory, the next step would be to determine transcript levels for Des9 in Tp_WT and Tp_Des9- lines using reverse transcription polymerase chain reaction (RT-PCR). This would establish if there was knock-down of *Des9*, with a resultant counter-balance of decreased Des9 activity by a functionally redundant protein, masking the knock-down of *Des9* by maintaining a "normal" FAMEs profile. It may therefore be that *T. pseudonana* cells require both proteins to be functioning optimally in order for optimal cell growth to occur.

## 4.4 CONCLUSIONS

Gene silencing was attempted based on a method previously proven successful in the diatom *P. tricornutum* (De Riso et al, 2009). Unfortunately the above study did not yield *T. pseudonana* cells with silenced native genes encoding Δ9-desaturase, Δ5-desaturase, Δ4-desaturase and Δ6-elongase activities. Interestingly, a similar experiment by colleagues utilising the same method in *P. tricornutum* to knock down genes involved in omega-3 biosynthesis also did not give rise to silenced cells (private communication). This may suggest that this method is neither the most efficient nor reproducible for generating knockout/down mutants to study omega-3 biosynthesis in diatoms.

Furthermore, for RNAi/siRNA functional genomics studies to be valuable and effective, specific silencing of any given target gene is essential, devoid of nonspecific knock-down and toxic side effects. This may not be the case in this study when the results for Tp_Des9- are taken into account, though further studies are needed to confirm the nature of the defect seen with this line. It is well established that knock-down by RNAi varies between experiments and laboratories, often having unpredictable off-target effects, and providing limited or temporary inhibition of gene function.

In order for gene silencing to be a useful tool for functional genomics studies in *T. pseudonana* it is necessary to have robust and reproducible protocols that specifically and

efficiently target a gene or DNA sequence in a complex genome, while avoiding the drawbacks of traditional methods outlined above. Alternative methods that could be investigated further in *T. pseudonana* include the use of site specific nuclease technologies to directly "edit" the genome. Such techniques include CRISPR/Cas methods (Cong et al, 2013; Hwang et al, 2013) and TALENs (Moscou and Bogdanove, 2009; Boch et al, 2009; Joung and Sander, 2012; Beurdeley et al, 2013). Genome editing is achieved using engineered nucleases composing sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module (Urnov et al, 2010; Carroll, 2011). These chimeric nucleases allow for efficient and precise genetic modifications by inducing targeted DNA double-strand breaks that stimulate cellular DNA repair mechanisms, including error-prone non-homologous end joining and homology-directed repair (Wyman and Kanaar, 2006). The ability to programme the DNA-binding domains derived from zinc- finger and transcription activator-like effector nucleases (ZFNs and TALENs) enables these methods to be extremely versatile.

It would be interesting to utilise these approaches to edit the genome of *T. pseudonana* in order to unravel some of the mysteries surrounding lipid metabolism in this diatom.

# Chapter 5

# General Discussion and Conclusions

## 5.1 SUMMARY OF MAJOR FINDINGS

### 5.1.1 Identification and Functional Characterisation of Novel Genes Encoding Omega-3 Biosynthetic Activities in Two Species of Microalgae

A database search carried out on the genomes of the unicellular photoautotrophic green alga *Ostreococcus* sp. RCC809 and cold-water diatom *Fragilariopsis cylindrus* led to the identification of two putative "front-end" desaturases (Δ6 and Δ4) from *Ostreococcus* RCC809, a Δ5-elongase from *Ostreococcus* RCC809 and one Δ6-elongase from *F. cylindrus*. *Ostreococcus* RCC809 and *F. cylindrus* hadn't previously been investigated for genes involved in their omega-3 biosynthetic pathways. Functional characterisation experiments in yeast revealed that the encoded enzyme activities efficiently convert their respective substrates (Table 11).

| Species | Designation | Defined Function | Conversion Rate (substrate) |
|---|---|---|---|
| *Ostreococcus* sp. RCC809 | **Ost809D6** | Δ6-desaturase | 54.1% (ALA) |
| *Ostreococcus* sp. RCC809 | **Ost809D4** | Δ4-desaturase | 15.1% (DPA) |
| *Ostreococcus* sp. RCC809 | **Ost809Elo5** | Δ5-elongase | 51.6% & 62.1% (ARA & EPA) |
| *F. cylindrus* | **FcElo6** | Δ6-elongase | 38.1% (GLA) |

**Table 11:** Characterised genes from *Ostreococcus* sp. RCC809 and *F. cylindrus*, defining enzyme function and conversion rates of specific substrates.

Of particular interest is the Δ6-desaturase from *Ostreococcus* RCC809, which demonstrated activity with specificity towards omega-3 substrates.

### 5.1.2 Characterisation of the FAMEs Profiles of *Ostreococcus* sp. RCC809 and *F. cylindrus*

The FAMEs profiles of these two previously uncharacterised microalgal species were analysed (Table 12). The strong preference for omega-3 versus omega-6 C18 substrates of the *Ostreococcus* Δ6-desaturase is likely reflected in its fatty acid profile, with accumulation of omega-6 substrate (LA) but not product (GLA), and the inverse accumulation of omega-3 product (SDA) but not substrate (ALA).

| | Composition (Molar %) ± SD | |
|---|---|---|
| **Fatty Acid** | ***Ostreococcus* sp. RCC809** | ***F. cylindrus*** |
| **16:0** | 20.12 ± 0.6 | 12.1 ± 0.2 |
| **16:1n-7** | 18.1 ± 0.3 | 24.5 ± 0.1 |
| **16:4n-3** | 5.08 ± 0.4 | ND |
| **18:0** | 1.24 ± 0.5 | 2.4 ± 0.3 |
| **18:1n-9** | 8.17 ± 0.2 | ND |
| **18:1n-7** | 8.03 ± 0.1 | ND |
| **LA** | 9.12 ± 0.2 | 0.7 ± 0.3 |
| **GLA** | 3.29 ± 0.3 | 1 ± 0.4 |
| **ALA** | 3.98 ± 0.4 | 0.8 ± 0.3 |
| **SDA** | 19.13 ± 0.6 | 5.7 ± 0.5 |
| **20:2n-6** | 0.65 ± 0.4 | 1 ± 0.2 |
| **20:4n-3** | ND | 7.4 ± 0.1 |
| **EPA** | 1.26 ± 0.4 | 31.4 ± 0.3 |
| **DHA** | 1.83 ± 0.5 | 2.5 ± 0.1 |
| **24:0** | ND | 1.4 ± 0.2 |

**Table 12:** Fatty acid composition of *Ostreococcus* sp. RCC809 and *F. cylindrus* cultures in stationary phase. Values are the average of three independent experiments (± standard deviation). ND—not detected.

The fatty acid profile of *F. cylindrus* revealed that the most abundant fatty acid in diatom was EPA (31.4% of TFA) followed by 16:1 and 16:0 (24.5% and 12.1% respectively). Like *Ostreococcus* sp. RCC809 it contained only low levels of DHA.

### 5.1.3 Optimisation of a PCR Method to Screen *T. pseudonana* transformants

This method enabled the timely and efficient screening of *T. pseudonana* transformants, avoiding the need to produce large culture volumes to extract the total DNA for subsequent screening by PCR.

### 5.1.4 Effect of Overexpressing Heterologous Genes Involved in the Omega-3 Biosynthetic Pathway on the FAMEs and Acyl-CoA Profiles of *T. pseudonana*

Preliminary results revealed markedly increased SDA, EPA and DHA production in transgenic *T. pseudonana*, as determined by FAMEs analysis, particularly for the line overexpressing a Δ6-desaturase encoding gene from *Ostreococcus tauri*. Changes were also observed in the acyl-CoA pools of transgenic lines. No deleterious impact on cell growth occurred. Further analyses over a period of two years unveiled a rhythmic cycle in the observed fatty acid profiles. This cycle was more pronounced in wild type *T. pseudonana* than in transgenic lines. Overexpression of genes encoding Δ6-desaturase and Δ6-elongase activities in *T. pseudonana* did have an impact on the FAMEs and acyl-CoA profiles, but the level of enhancement was skewed by the significant variations observed in the wild type. Further investigation is required to determine the source of this variation in wild type *T. pseudonana* cells, which may result in the need for a different approach to the enhancement of EPA and DHA production and accumulation in *T. pseudonana*.

### 5.1.5 Altered Lipid Droplet and Chloroplast Phenotype in *T. pseudonana* Overexpression Genes Involved in Omega-3 Biosynthesis

The images obtained from confocal microscopy studies demonstrated a clear visual difference between wild type and transgenic cells. The precise reasons for the diverse chloroplast and lipid droplet morphologies are unclear; however, one explanation could be the increase in longer chain fatty acids (>C18) in the acyl-CoA pools, resulting in altered membrane lipid composition and a consequential phenotype. A total lipid analysis and further investigations would be required to determine if this is the source of altered chloroplast morphology observed in transgenic *T. pseudonana* cells.

### 5.1.6 Altered Growth Phenotype of *T. pseudonana* Cells targeted for the Knock-down of Δ9-desaturase Activity

Tp_Des9- lines exhibited a significantly different growth phenotype to that of wild type *T. pseudonana* whilst maintaining a "normal" fatty acid profile. The logical explanation for these observations is that there was a knock-down effect on the target Δ9-desaturase and its activity, but this was masked by increased activity of another, functionally redundant, protein that escaped sequence-based silencing. It may therefore be that *T. pseudonana* cells require both proteins to be functioning optimally in order for optimal cell growth to occur. Further research is required to determine the processes behind the growth phenotype in Tp_Des9- cells.

## 5.2 DISCUSSION

The novel genes identified in Chapter 2 expand the repository of genes available for metabolically engineering omega-3 biosynthetic pathways, be that in plants, algae or other oleaginous microorganisms. Sequence alignments with other desaturases with similar sequence identities revealed that amino acid sequence alone cannot be used to determine enzyme substrate specificity. Enzymes with similar function can be structurally and phylogenetically diverse (Qi et al, 2002; Meyer et al, 2004), compounding the difficulty of assigning function to genes following genome sequencing. Such a task is even further complicated in diatoms which are predicted to have >10,000 genes, the majority of which show no homology to genes of known function (Ashworth et al, 2013). Moreover, during the course of this PhD a number of other putative desaturase and elongase coding genes, from a variety of algal species were tested (data not shown), based on their sequence homology with a number of fully characterised genes from plants and other algae. These were not found to be functional, despite being tested on a wide range of potential substrates, including those for other classes of desaturase/elongase enzymes. Such results further underline the difficulty in identifying gene function in these organisms.

The unique evolutionary history of diatoms contributes to their complex nature, which makes them fascinating to study, but complicates complete understanding of the processes governing and regulating their physiology and adaptive mechanisms. Diatoms exhibit an array of regulatory mechanisms to maintain homeostasis and adapt to their environment (Armbrust et al, 2004; Ashworth et al, 2013). The regulation and level of gene expression in these processes is innately connected with circadian/diurnal and seasonal cycles (Johnson et

al, 1991; Jacobshagen and Johnson, 1994; Fábregas et al, 2002; Golden and Canales, 2003; Depauw et al, 2012; Braun et al, 2014), which can occur in an anticipatory manner (Ashworth et al, 2013). The observations of non-transcriptional clocks in algae (Bouget et al, 2014) reveal another layer of complexity.

In Chapter 3 the observations regarding the overexpression of a selection of genes highlighted the complexities surrounding the metabolic engineering of the diatom *T. pseudonana*. The results of this study suggest that an alternative approach may be required to enhance omega-3 production and accumulation in this diatom. It would be valuable to carry out RT-PCR on both overexpression and knock-down lines reported above to provide further correlation (or not) between observed fatty acid profiles and the activity/inactivity of the gene of interest.

Moreover, full lipidome analyses of overexpression and knock-down lines may uncover explanations for the phenotypic variations described above. This may also lead to the identification of further targets to engineer with the goal of improving omega-3 fatty acid production and accumulation in the diatom *T. pseudonana*.

## 5.3 CONCLUSIONS AND FUTURE PERSPECTIVES

The advancement of our understanding of microalgal lipid metabolism, including the characterisation of genes involved in biosynthetic pathways and the factors regulating them, will enable the development of industrially compatible large-scale sustainable systems for the production of value added products, such as omega-3 fatty acids. The results and observations provided in this thesis contribute new valuable information to this field of research.

Further research will provide beneficial insights and bring us closer to fully understanding lipid metabolism in microalgae. Transcriptional analyses will highlight the relative activity of genes involved in omega-3 biosynthesis, highlighting potential bottlenecks and even possible alternative routes to desirable products. Genome editing technologies may prove to be particularly helpful in uncovering gene function as well as enabling targeted gene addition.

Additionally, the development of more efficient transformation technologies utilising multi-gene vectors will expand the repertoire of genetic engineering possibilities. Moreover, a shift towards a synthetic biology approach of rational engineering, coupled with systems biology,

may enable the design and development of a "gold-standard" of omega-3 fatty acid producing microalgal species.

Another consideration that will require research and development is the upscaling of engineered strains in order for them to be industrially and commercially viable.

There is still a long way to go before the mysterious world of the diatom is fully unlocked, but with the exponentially expanding field of research into microalgae and development of high-throughput technologies, we will get closer to fully understanding these fascinating organisms.

# Chapter 6

# Materials and Methods

## 6.1 GROWTH AND HARVESTING OF MICROALGAL STRAINS

### 6.1.1 *Ostreococcus* sp. RCC809

Cultures of *Ostreococcus* sp. RCC809 were grown in ESAW medium (Harrison et al, 1980; Berges et al, 2001) under constant temperature and illumination at 20°C and 20 µM photons $m^{-2}$ $s^{-1}$ under white fluorescent light. Cultures were agitated manually every two days to prevent aggregation. Harvested cells were collected by centrifugation at 3500g (RCF or G-force (g) = 1.12 x R x (RPM/1000)², where R = radius of rotation in millimetres).

### 6.1.2 *Fragilariopsis cylindrus*

Cell pellets of *F. cylindrus* were provided by Dr Jan Strauss at UEA. Cultures of *F. cylindrus* were grown in Aquil media (Morel et al, 1979; Price et al, 1989; Sunda et al, 2005) at 4°C under illumination at 40 µM photons $m^{-2}$ $s^{-1}$, and were shaken manually from time to time. Harvested cells were collected by centrifugation.

### 6.1.3 *Thalassiosira pseudonana*

Cultures of *T. pseudonana* (strain CCMP1335) were grown in ESAW medium (Harrison et al, 1980) at 20°C with under white fluorescent lights in constant illumination (60 µmol photons $m^{-2}$ $s^{-1}$). Analyses of the wild type and transgenic algae were performed during stationary growth phases. Growth stage was determined by cell counting (Nexcelom Bioscience, Lawrence, MA, USA) with reference to growth curves measured for Tp_WT and transgenic cells at 20°C. Harvested cells were collected by centrifugation at 3500g.

### 6.1.4 Growth Curve Analysis of Microalgal Strains

Cells were counted and growth/cell characteristics were analysed using a Cellometer Auto X4 Cell Counter (Nexcelom Bioscience, Lawrence, MA, USA).

## 6.2 IDENTIFICATION AND CLONING OF PUTATIVE PUFA GENES

Genomes of the unicellular alga *Ostreococcus* sp. RCC809 and the marine diatom *Fragilariopsis cylindrus* were analysed with BLAST (Altschul et al, 1990) using N-terminal cytochrome b5-fusion desaturases and ELO-like elongating activity as templates. This analysis revealed the presence of several genes coding for putative PUFA desaturases and elongases. The putative Δ6- and Δ4-desaturase and Δ5-elongase sequences from *Ostreococcus* sp. RCC809 and Δ6-elongase sequence from *F. cylindrus*, were used as templates to chemically synthesise (Genscript Corporation, Piscataway, NJ, USA) codon-optimised nucleotide sequences for expression in diatoms. The codon-optimised genes were subcloned into the *KpnI-SacI* sites present in the galactose inducible yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA). Expression vectors were transformed and maintained in *E. coli* (Competent JM109 Cells, Promega, Madison, WI, USA) and plasmids were isolated by established methods (Bimboin and Doly, 1979) using a commercial kit (Qiagen, Venlo, Netherlands).

## 6.3 FUNCTIONAL EXPRESSION AND CHARACTERISATION IN YEAST

1ml of overnight liquid yeast culture (*Saccharomyces cerevisiae* strain W303-1A, 100ml YPD, 30°C, 210 rpm) was harvested (3000g x 5 minutes) and plasmids containing ORFs encoding putative desaturation and elongation activities were introduced by a lithium acetate method (Elble, 1992). 10µl plasmid DNA and 8µl carrier DNA (10mg/ml herring sperm DNA) were added to the cell pellet from the overnight culture and vortexed for 10 seconds, followed by the addition of 500µl PLATE buffer (45ml 45% PEG 4000 (w/v) (autoclaved), 5ml 1M filter-sterilised lithium-acetate, 0.5ml 1M Tris-HCl (pH 7.5) and 0.1ml 0.5M EDTA). The solution was vortexed again and incubated at room temperature for 24 hours. Following incubation, cells were pulsed and PLATE buffer was removed. The cells were then resuspended in 40µl $H_2O$ and plated on selective plates (synthetic dextrose minimal medium minus uracil (SD-Ura)) and incubated for 2 – 3 days (or until colonies appeared) at 30°C. Positive transformants were used to inoculate cultures and were grown overnight in SD-Ura liquid medium at 30°C and 210rpm in the presence of 2% (w/v) raffinose. Overnight cultures were used to inoculate 20ml SD-Ura in the presence of 2% (w/v) raffinose and 1% (w/v) tergitol-Nonidet P-40 (Sigma-Aldrich, Haverhill, UK) and grown until an $OD_{600}$ of 0.2 – 0.4 was reached (Mitchell and Martin, 1995). The 30ml culture was split into 2 x 15ml cultures and one was induced by the addition of galactose to 2% (w/v) in the presence of 0.5M of the corresponding fatty acid.

The second 15ml culture was used as a control and "negatively induced" by the addition of glucose to 1% (w/v) in the presence of 0.5M of the corresponding fatty acid. Both cultures were grown at 22°C for 48 hours and then taken forward for FAMEs analysis.

## 6.4 ANALYSIS OF FATTY ACID METHYL ESTERS (FAMES)

### 6.4.1 FAMEs Analysis of Yeast

Fatty acids were extracted and methylated as described previously (Sayanova et al, 1997). Washed cell pellets were dried under $N_2$ gas, followed by the addition of 1ml methylation mix (85ml methanol, 5ml 2,2-dimethoxypropane, 10ml HCl (for 100ml methylation mix)) and incubation at 85°C for 1 hour. After incubation 500µl NaCl and 500µl hexane were added to the sample, which was then shaken and left until the two phases separated. The upper hexane phase was removed with a glass pipette and transferred to a clean vial, followed by concentration under $N_2$ gas and the addition of 50µl hexane for subsequent analysis. Total fatty acids were analysed by gas chromatography coupled with a flame ionisation detector (GC-FID) of methyl ester derivatives. FAME samples were analysed by liquid chromatography using a Hewlett-Packard 6890 series Gas Chromatograph and an Alltech AT-225 (30m × 0.32mm × 0.3µm) capillary column. Inlet and detector temperature was set to 250°C and 1µL of each sample was analysed using splitless injection and a constant flow rate of 2ml/min. The oven temperature cycle was set a follows: A start temperature of 50°C was held for 1 minute to allow vaporised samples and the solvent (hexane) to condensate at the front of the column. Oven temperature was then increased rapidly to 190°C at a rate of 40°C/min followed by a slower increase to 220°C at a rate of 1.5°C/min. The final temperature of 220°C was held for 1 min giving a total run time of 25 minutes and 50 seconds per sample. FAMEs were detected using a flame ionisation detector (FID). Chromatograms were analysed using the Agilent ChemStation software Rev B.04.02 (118). Peak area percentages (area %) were converted to molecular percentages (mol %) to correct the error inherent to FID due to the different carbon number of each compound.

### 6.4.2 FAMEs Analysis of Microalgal Strains

Algal cells were harvested from mid-stationary phase cultures by centrifugation at 3500g for 5 min. Lipids were extracted and methylated as described (Garces and Mancha, 1993) with minor modifications. A 15ml aliquot of algal culture was harvested and the cell pellet was

dried under $N_2$ gas to remove excess $H_2O$, followed by the addition of 900µl methylation mix (methanol : toluene : 2,2-dimethoxypropane : $H_2O$ in the ratio 33 : 14 : 2 : 1) and 900µl heptane and incubation at 80°C for two hours. Following methylation the heptane fraction was concentrated under $N_2$ gas and re-suspended in 40µl solvent (heptane) prior to injection of 1µl on to the GC column. Methyl ester derivatives of total fatty acids extracted were analysed by GC (Agilent 7890A) using an Agilent DB-225 column (30m × 0.32mm × 0.3µm). Inlet and detector temperature was set to 250°C and 1µl of each sample was analysed using splitless injection and a constant flow rate of 2ml/min. The oven temperature cycle was set as follows: a start temperature of 50°C was held for 1 min to allow vaporised samples and the solvent (hexane) to condensate at the front of the column. Oven temperature was then increased rapidly to 190°C at a rate of 40°C/min followed by a slower increase to 220°C at a rate of 1.5°C/min. The final temperature of 220°C was held for 1 min giving a total run time of 25 minutes and 50 seconds per sample. FAMEs were detected using a Flame Ionisation Detector (FID). Chromatograms were analysed using the offline session of the Agilent ChemStation software. Peak area percentages (area %) were converted to molecular percentages (mol %) to correct the error inherent to FID due to the different carbon number of each compound. The retention time and identity of each FAME peak was calibrated using the FAME Mix Rapeseed oil standard (Supelco, Sigma-Aldrich, Haverhill, UK) supplemented with 2% (w/w) methyl 11,14-eicosadienoate (C20:2n-6). 1mM methyl heptadecanoate (C17:0) was added to samples as an internal standard.

## 6.5 PREPARATION OF CONSTRUCTS FOR TRANSFORMATION IN *T. PSEUDONANA*

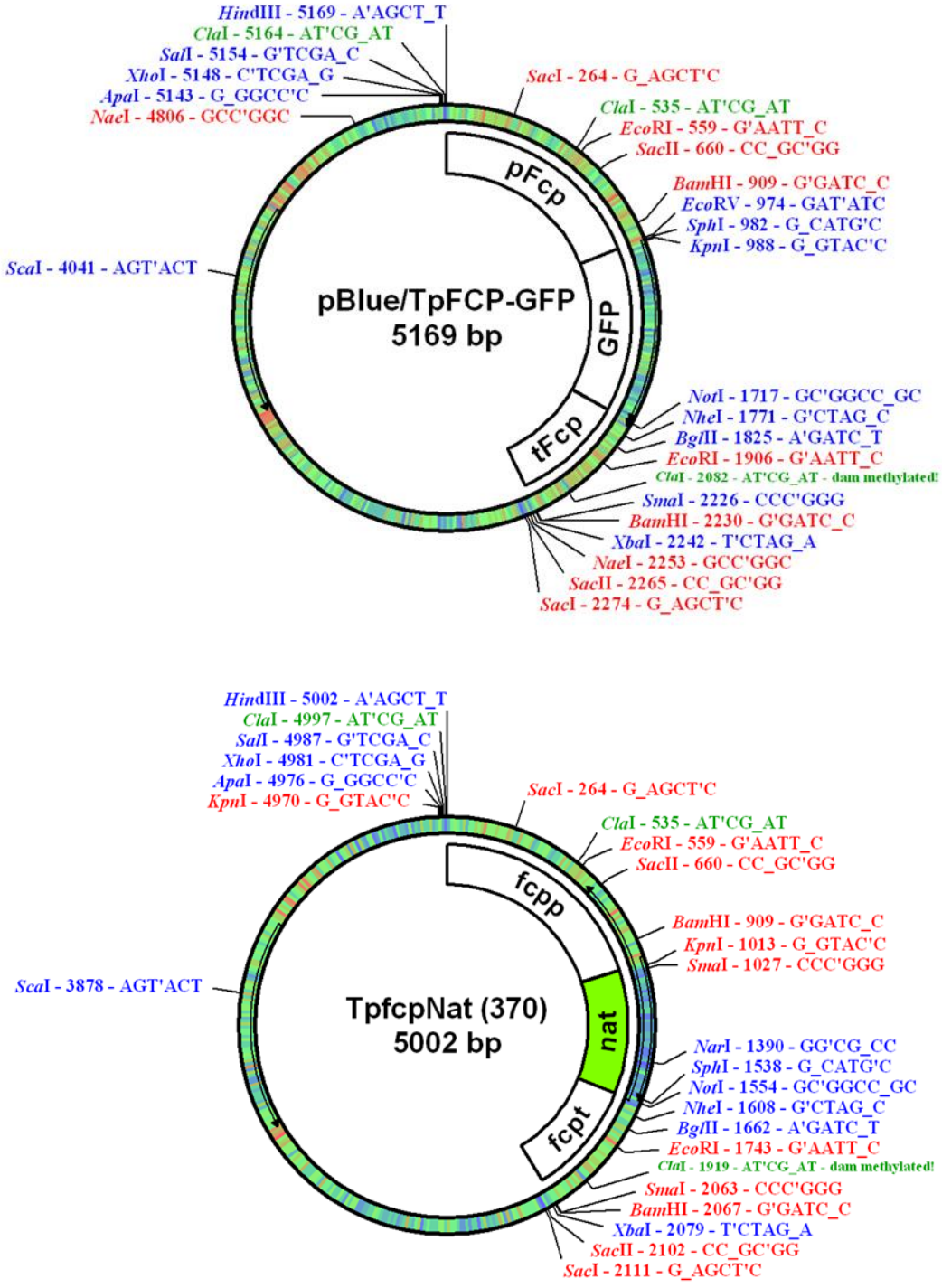



**Figure 37:** Transformation vectors for *T. pseudonana* as developed by Poulsen et al, 2006. Top is the constitutive pTpFCP-GFP plasmid containing the *fcp* promoter and GFP tag. Below is the pTpFCP-Nat plasmid containing the constitutive *fcp* promoter and *nat* gene conferring resistance to the antibiotic nourseothricin (clonNAT). Plasmids must be co-transformed into *T. pseudonana*.

### 6.5.1 Overexpression Constructs

The coding regions of *Ostreococcus tauri* Δ6-desaturase (Tonon et al, 2005a), *Ostreococcus* sp. RCC809 Δ6-desaturase (Vaezi et al, 2013), *F. cylindrus* Δ6-elongase (Vaezi et al, 2013), *O. tauri* Δ5-elongase (Pereira et al, 2004) and *Ostreococcus* sp. RCC809 Δ5-elongase (Chapter 2) were used as templates to chemically synthesise (Genscript Corporation, Piscataway, NJ, USA) codon-optimised nucleotide sequences for expression in *T. pseudonana*. These sequences were inserted as *EcoRV-KpnI* fragments into the pTpFCP-GFP vector (Figure 37) and co-transformed with pTpFCP-Nat in *T. pseudonana* (Poulsen et al, 2006). Expression vectors were transformed and maintained in *E. coli* (Competent JM109 Cells, Promega, Madison, WI, USA) and plasmids were isolated by established methods (Bimboin and Doly, 1979) using a commercial kit (Qiagen, Venlo, Netherlands).

### 6.5.2 Inverted Repeat Constructs

Inverted repeat constructs were generated using standard molecular cloning procedures (Sambrook et al, 1989) as described previously (De Riso et al, 2009). The Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) was used to amplify a whole genome sample from *T. pseudonana*. This was used as a template for the construction of inverted repeat sequences targeting native genes encoding Δ9-desaturase (Accession number: XM_002288140), Δ5-desaturase (Accession number: XM_002288806.1), Δ4-desaturase (Accession number: AY817156.1) and Δ6-elongase (Accession number: AY591337.1). Target sequences were found using Target Finder (Genscript Corporation, Piscataway, NJ, USA), using default settings. Primers are shown in Table 14 and were checked for complex loops and Holliday junctions using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc., Coralville, IA, USA), using default settings. Sense and antisense sequences were generated as *EcoRV-HpaI* (sense) and *HpaI-KpnI* (antisense) fragments for ligation to each other and subsequent insertion as *EcoRV-KpnI* fragments into the pTpFCP-GFP vector for transformation in *T. pseudonana*. Primers D9SFwd, D9ARev (for Δ9-desaturase), D5SFwd, D5ARev (for Δ5-desaturase), D4SFwd, D4ARev (for Δ4-desaturase), E6SFwd and E6ARev (for Δ6-elongase) were used to screen transformants. Positive transformants were taken forward for FAMEs and growth curve analysis.

Expression vectors were transformed and maintained in *E. coli* (Competent JM109 Cells, Promega, Madison, WI, USA) and plasmids were isolated by established methods (Bimboin and Doly, 1979) using a commercial kit (Qiagen, Venlo, Netherlands).

## 6.6 BIOLISTIC TRANSFORMATION OF *T. PSEUDONANA*

Biolistic transformation of *T. pseudonana* was performed using established methods for this diatom (Poulsen et al, 2006). 50µl of 0.6µm gold carrier particles (Bio-Rad Laboratories, Richmond, CA, USA) were coated with 5µg of plasmid DNA containing the gene of interest and 5µg of plasmid DNA containing antibiotic resistance for co-transformation into *T. pseudonana* with 50µl 2.5M CaCl and 20µl 0.1M spermidine (free base). Three independent biolistic transformations (PDS-1000 He System and all accessories, Bio-Rad Laboratories, Richmond, CA, USA) were carried out at 1100psi, 1350psi and 1550psi and combined in the next step. Bombarded cells were transferred to 75ml liquid ESAW medium and incubated overnight at 20°C with constant illumination (100µmol $m^{-2}$ $s^{-1}$). Overnight liquid culture was split into 3 and centrifuged at 3000g for 10 minutes at 4°C. Cells were plated on 50% ESAW (v/v) 1.2% agar (w/v) plates containing 100µg/ml nourseothricin (clonNAT, WERNER BioAgents, Jena, Germany). The clonNAT plates were incubated in 24 hour light under fluorescent lights (100µmol $m^{-2}$ $s^{-1}$) and at 20°C until colonies appeared (approx. 3 – 4 weeks). Selected clonNAT-resistant colonies were transferred to fresh 1.5ml liquid ESAW plus 100µg/ml clonNAT in microtitre plates before being transferred to 150ml liquid ESAW medium plus 100µg/ml clonNAT after 5 days.

## 6.7 ACYL-COA PROFILING OF *T. PSEUDONANA*

Algal cells were harvested from mid-stationary phase cultures by centrifugation (3500g, 5 minutes), frozen in liquid nitrogen and extracted as previously described (Larson and Graham, 2001) for reverse-phase liquid chromatography (LC) (Agilent 1200 LC system; Gemini C18 column, 2mm inner diameter, 150mm with 5mm particles) with electrospray ionisation tandem mass spectrometry (multi reaction monitoring (MRM)) in positive ion mode (AB4000 Q-TRAP (Applied Biosystems), spray voltage set to 5kV, nebulising gas at 40psi, focusing gas at 40psi and curtain gas at 20psi, source temperature was held at 750°C). LC-MS/MS+MRM analysis followed the methods described by Haynes et al (2008). Acyl-CoAs were identified by MRM and precursor-produced ion pairs of specific acyl-CoAs were followed. For the purpose of identification and calibration, standard acyl-CoA esters with acyl chain lengths from C14 to C20 were used, either as free acids or lithium salts. (Sigma-Aldrich, Haverhill, UK).

## 6.8 OPTIMISED COLONY PCR METHOD FOR SCREENING *T. PSEUDONANA* TRANSFORMANTS

In order to confirm the presence of a transgene a sample was taken from each culture and screened using an optimised "colony PCR" method based on DNA extraction using Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA)(Walsh et al, 1991). A number of variations on this method were experimented with until an optimal and reproducible protocol was found. Each culture was screened as per this final protocol. A 10ml sample from a stationary phase culture was centrifuged at 3000g for 5 minutes and the supernatant was discarded. The cell pellet was transferred to a glass vial in 20µl sterile $H_2O$ to which 20µl 100% ethanol was added. This solution was then vortexed to mix followed by the addition of 200µl 6% Chelex-100 (w/v). The solution was vortexed again and incubated at 98°C for 10 minutes. Following incubation the solution was centrifuged at 3000g for 10 minutes and 1µl of the supernatant was used per PCR reaction. Cultures testing positive for a transgene or inverted repeat fragment were taken forward for FAMEs profiling and growth curve analysis.

## 6.9 CONFOCAL MICROSCOPY

### 6.9.1 Localisation of GFP-Tagged Transgenic Protein in Tp_OtD6GFP

*T. pseudonana* cells expressing GFP-tagged *O. tauri* Δ6-desaturase were harvested during the exponenetial growth phase and immobilised on slides with a 1% agar ESAW medium. Confocal fluorescence microscopy imaging was performed on transgenic and wild type cells using the Zeiss LSM 780 confocal laser scanning microscope (Jena, Germany). GFP fluorescence (Argon laser, 488nm) was detected using a 505/550nm bandpass filter. For dual imaging, chloroplast auto-fluorescence (HeNe laser, 543nm) was detected with a 585nm long pass filter in the multitrack mode of the microscope.

### 6.9.2 Visualisation of Chloroplasts and Lipid Droplets in Wild Type and Transgenic *T. pseudonana* lines

*T. pseudonana* cells overexpressing genes encoding omega-3 biosynthetic activities were harvested during the stationary growth phase and centrifuged at 2000g for 7 minutes. Preliminary work found that BODIPY staining was more effective than Nile Red for the visualisation of lipid droplets in *T. pseudonana*. Cell pellets were stained with 40µg BODIPY

(stored at 1µg/µl under DMSO) (Life Technologies, Paisley, UK) by homegenisation with the cell pellet followed by incubation in the dark at room temperature for 20 minutes. Samples were spun down, supernatant discarded and resuspended in 500µl room temperature liquid ESAW. Confocal fluorescence microscopy imaging was performed on transgenic and wild type cells using the Zeiss LSM 780 confocal laser scanning microscope (Jena, Germany). BODIPY fluorescence (Argon laser, 488nm) was detected using a 505/550nm bandpass filter. For dual imaging, chloroplast auto-fluorescence (HeNe laser, 543nm) was detected with a 585nm long pass filter in the multitrack mode of the microscope.

## 6.10 STATISTICAL ANALYSIS

### 6.10.1 ANOVA

To determine the level of variance between technical and biological repeats a one-way analysis of variance (ANOVA) was carried out to test the null hypothesis that samples from technical and biological replicate groups were drawn from populations with the same mean values. F < F crit in all samples tested, so the null hypothesis was accepted, there was no significant variation in the means from technical and biological repeats.

### 6.10.2 T-Test

A student T-test was used to determine if the variation of the data obtained from FAMEs analysis of overexpression lines was statistically significant between and within lines. Variation determined to be statistically significant is discussed in Chapter 3.

## 6.11 PCR PRIMERS

### 6.11.1 Primers Used in Characterisation and Overexpression Studies

| Target Gene | Amplicon Size (bp) | Primer Name | Primer Sequence |
|---|---|---|---|
| *O. tauri* Δ6-desaturase (Tonon et al, 2005a) | 1371 | OtD6-FCP Fwd<br>OtD6-FCP Rev<br>OtD6-FCP GFP Rev | 5'-**gatatcaccaaa**atgtgcgtggagacgg-3'<br>5'-*ggtacc*aatttacgccgtctttccggag-3'<br>5'-*ggtacc*ttacgccgtctttccggag-3' |
| *Ostreococcus* sp. RCC809 Δ6-desaturase (Vaezi et al, 2013) | 1430 | Ost809 Des6Opt Fwd<br>Ost809 Des6Opt Rev<br>Ost809 Des6Opt GFP Rev | 5'-**gatatcaccaaa**atgcgtgtggaaaccg-3'<br>5'-*ggtacc*ctaaaccgtcttctttgtgatttg-3'<br>5'-*ggtacc*aaccgtcttctttgtgatttg-3' |
| *Ostreococcus* sp. RCC809 Δ4-desaturase (Vaezi et al, 2013) | 1431 | Ost809 Des6Opt Fwd<br>Ost809 Des6Opt Rev | 5'-atgccaactactcgttctcg-3'<br>5'-ttaagcagatttttttagattgac-3' |
| *F. cylindrus* Δ6-elongase (Vaezi et al, 2013) | 863 | Frag Elo6Opt Fwd<br>Frag Elo6Opt Rev<br>Frag Elo6Opt GFP Rev | 5'-**gatatcaccaaa**atggatgagtacaaggcc-3'<br>5'-*ggtacc*cttcaggctgttggacttcttg-3'<br>5'-*ggtacc*ggctgttggacttcttg-3' |
| *O. tauri* Δ5-elongase (Pereira et al, 2004) | 945 | OtElo5 Opt Fwd<br>OtElo5 Opt Rev<br>OtElo5 Opt GFP Rev | 5'-**gatatcaccaaa**ggtaccaagcttg-3'<br>5'-*ggtacc*cctcgagggtaccgag-3'<br>5'-*ggtacc*cgagggtaccgag-3' |
| *Ostreococcus* sp. RCC809 Δ5-elongase (Chapter 2) | 926 | O809Elo5 Opt Fwd<br>O809Elo5 Opt Rev<br>O809Elo5 Opt GFP Rev | 5'-**gatatcaccaaa**atgggaacaagagtg3'<br>5'-*ggtacc*cttaattaatcaatccac-3'<br>5'-*ggtacc*ggattaatcaatccac-3' |

**Table 13:** Primers used during this PhD. **Bold** represents *EcoRV* sites and *italics KpnI* sites. GFP Rev primers include readthrough to GFP, other Rev primers do not.

### 6.11.2 Primers Used to Construct Inverted Repeat Fragments

| Fragment | Amplicon Size (bp) | Primer Name | Primer Sequence |
|---|---|---|---|
| Des9 Sense | 458 | D9SFwd | 5'-**gatatcaccaaa**aaagaccaggagccctcatt-3' |
| Des9 Sense | 458 | D9SRev | 5'- GTTAACgtccatatgattggtgtacc-3' |
| Des9 Anti | 272 | D9AFwd | 5'-*ggtacc*aataaagaccaggagccctcatt-3' |
| Des9 Anti | 272 | D9ARev | 5- GTTAACcgagtaggctgtccagaagg-3' |
| D5 Sense | 472 | D5SFwd | 5'-**gatatcaccaaa**tcatcgcaattgtgtttggt-3' |
| D5 Sense | 472 | D5SRev | 5'- GTTAACggggctgacaatgttcaagt-3' |
| D5 Anti | 355 | D5AFwd | 5'-*ggtacc*aattcatcgcaattgtgtttggt-3' |
| D5 Anti | 355 | D5ARev | 5- GTTAACtcaatgaattgcggattgaa-3' |
| D4 Sense | 417 | D4SFwd | 5'-**gatatcaccaaa**acattaccgactttgcgtcc-3' |
| D4 Sense | 417 | D4SRev | 5'- GTTAACgttgaatacacgtgccgatg-3' |
| D4 Anti | 225 | D4AFwd | 5'-*ggtacc*aatacattaccgactttgcgtcc-3' |
| D4 Anti | 225 | D4ARev | 5- GTTAACgctccacaaccctcctcttc-3' |
| Elo6 Sense | 531 | E6SFwd | 5'-**gatatcaccaaa**cacaacatgcatttgaaggg-3' |
| Elo6 Sense | 531 | E6SRev | 5'- GTTAACaggaatttgatggggtaggg-3' |
| Elo6 Anti | 270 | E6AFwd | 5'-*ggtacc*aatcacaacatgcatttgaaggg-3' |
| Elo6 Anti | 270 | E6ARev | 5- GTTAACgctcacctctctatcggcac-3' |

**Table 14:** Primers for inverted repeat constructs. **Bold** represents *EcoRV* sites, CAPITALS *HpaI* sites, *italics KpnI* sites and STOP codon is underlined. STOP prevents readthrough and expression of GFP.

# Appendix

## APPENDIX I) PAPER (VAEZI ET AL, 2013) BASED ON CHAPTER 2, PUBLISHED IN THE JOURNAL MARINE DRUGS

*Mar. Drugs* **2013**, *11*, 5116-5129; doi:10.3390/md11125116

OPEN ACCESS

***marine drugs***

**ISSN 1660-3397**

www.mdpi.com/journal/marinedrugs

*Article*

**Identification and functional characterisation of genes encoding enzyme activities involved in omega-3 polyunsaturated fatty acid biosynthesis from unicellular microalgae**

**Royah Vaezi[1], Johnathan A. Napier[1] and Olga Sayanova[1,*]**

[1] Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK; E-Mails: royah.vaezi@rothamsted.ac.uk (R.V.), johnathan.napier@rothamsted.ac.uk (J.N.), olga.sayanova@rothamsted.ac.uk (O.S.)

***** Author to whom correspondence should be addressed; E-Mail: olga.sayanova@rothamsted.ac.uk (O.S.); Tel.: +44-(0)1582-763133 Ext 2134; Fax: +44-(0)1582-763010.

*Received: 22 September 2013; in revised form: 20 November 2013 / Accepted: 26 November 2013 / Published: 16 December 2013*

---

**Abstract:** In order to identify novel genes encoding enzymes involved in the biosynthesis of nutritionally important omega-3 long chain polyunsaturated fatty acids, a database search was carried out in the genomes of the unicellular photoautotrophic green alga Ostreococcus RCC809 and cold-water diatom Fragilariopsis cylindrus. The search led to the identification of two putative "front-end" desaturases (Δ6 and Δ4) from Ostreococcus RCC809 and one Δ6-elongase from F. cylindrus. Heterologous expression of putative open reading frames (ORFs) in yeast revealed that the encoded enzyme activities efficiently convert their respective substrates: 54.1% conversion of α-linolenic acid for Δ6-desaturase, 15.1% conversion of 22:5n-3 for Δ4-desaturase and 38.1% conversion of γ-linolenic acid for Δ6-elongase. The Δ6-desaturase from Ostreococcus RCC809 displays a very strong substrate preference resulting in the predominant synthesis of stearidonic acid (C18:4Δ6,9,12,15). These data confirm the functional characterization of omega-3 long chain polyunsaturated fatty acid

biosynthetic genes from these two species which have until now not been investigated for such activities. The identification of these new genes will also serve to expand the repertoire of activities available for metabolically engineering the omega-3 trait in heterologous hosts as well as providing better insights into the synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in marine microalgae.

**Keywords:** microalgae; omega-3 polyunsaturated fatty acid; desaturases; elongases

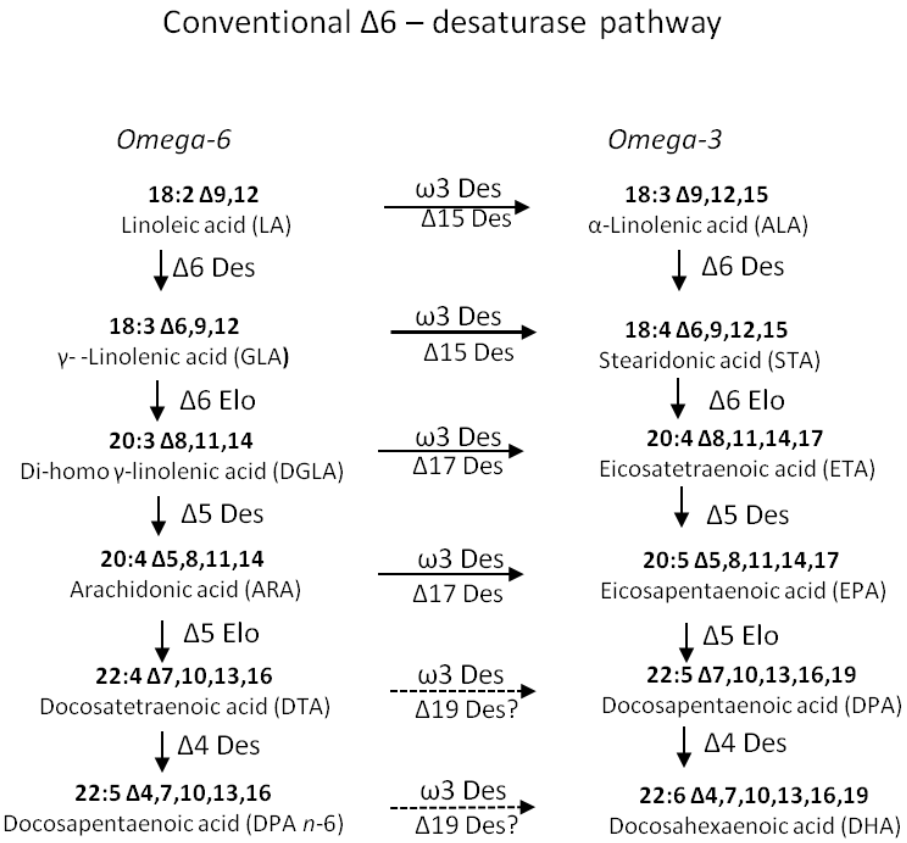
## 1. Introduction

It is now well accepted that omega-3 long chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6Δ4,7,10,13,16,19) are vital for human health and nutrition and play a crucial role in preventing cardiovascular diseases and associated precursor conditions such as metabolic syndrome and obesity [1,2]. Currently, oily marine fish is the major dietary source of these fatty acids; however, considering growing pressure on global fish stocks and pollution of the marine environment there is an urgent need for an alternative cost-effective solution for large-scale production of LC-PUFAs.

In recent years, the feasibility of using higher plants for the production of omega-3 LC-PUFAs has been explored and considerable progress has been made in effective production of EPA and DHA in oilseeds [3–5]. A variety of strategies have been used to introduce (via genetic engineering) the omega-3 LC-PUFA metabolic pathways in oil crops, mainly by expressing desaturase and elongase genes involved in different biosynthetic routes for EPA and DHA accumulation [6]. Marine algae are the primary producers of omega-3 LC-PUFAs and therefore represent the logical source for the identification of genes encoding the enzymes required for the synthesis of EPA and DHA. Most omega-3 LC-PUFA-synthesising marine organisms utilize the so-called Δ6-desaturase "conventional" aerobic pathway which relies on a consecutive series of altering desaturation and elongation steps to convert α-linolenic acid (ALA; 18:3Δ9,12,15) to EPA and DHA (Figure 1). The first step in this pathway is the Δ6-desaturation of both linoleic acid (LA; 18:2Δ9,12) and ALA, resulting in the synthesis of γ-linolenic acid (GLA; 18:3Δ6,9,12) and stearidonic acid (SDA; 18:4Δ6,9,12,15), respectively. This step is followed by a Δ6-specific C2 elongation, yielding di-homo γ-linolenic acid (DGLA; 20:3Δ8,11,14) and eicosatetraenoic acid (ETA; 20:4Δ8,11,14,17). Finally, these LC-PUFAs are desaturated by a Δ5-desaturase to generate arachidonic acid (ARA; 20:4Δ5,8,11,14) and EPA, respectively. In DHA-accumulating microorganisms, the pathway involves C2 elongation of EPA to docosapentaenoic acid (DPA; 22:5 Δ7,10,13,16,19) by a specific Δ5-elongase which is then desaturated by a Δ4-specific desaturase to yield DHA. Although most enzymes involved in this pathway show limited discrimination between n-3 and n-6 acyl-substrates, the predominant presence of n-3 LC-PUFAs in most marine microorganisms indicates the likely presence of ω-3-desaturases which convert omega-6 to

omega-3. However, some examples of Δ6-desaturases cloned from microalgae show a preference for ω-3 substrates (as was reported for higher plant Δ6-desaturases from Primula spp and Echium [7–9]. An ω-3 substrate preference has previously been described for enzymes from Mantoniella squamata [10], Micromonas pusilla [11] and Ostreococcus lucimarinus [12].

**Figure 1.** Pathway for the biosynthesis of LC-PUFAs in microalgae




In this present study, we examined the fatty acid profile of two marine microalgae, the unicellular photoautotrophic green alga Ostreococcus RCC809 and the cold-water diatom Fragilariopsis cylindrus, both of which have previously only been subject to limited investigation as to the nature of their synthesis and accumulation of omega-3 LC-PUFAs. We also used on-going genomic sequencing projects for these two organisms to identify and functionally characterize three examples of genes involved in the biosynthesis of EPA and DHA. Interestingly, one particular enzyme (Δ6-desaturase from Ostreococcus RCC809) showed a strong preference of ω-3 substrates against ω-6.

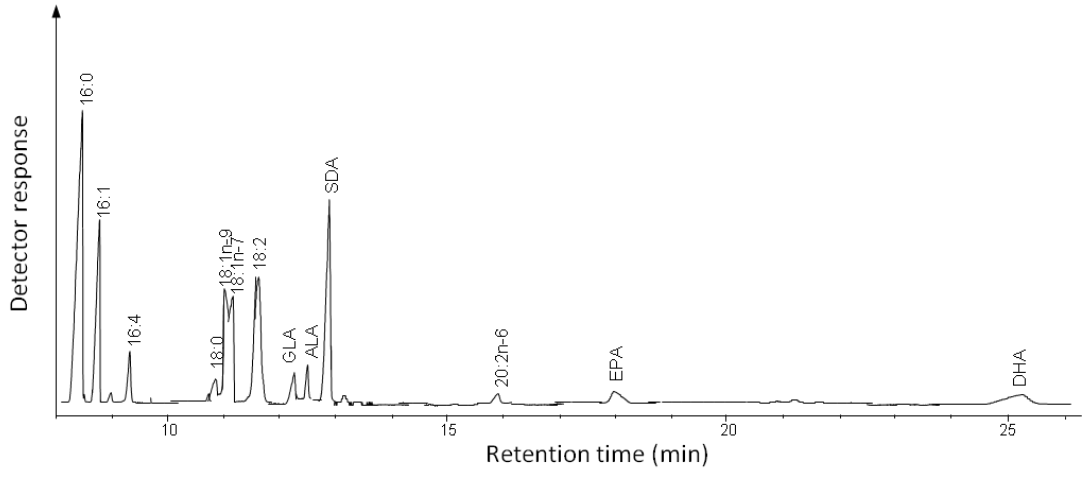
## 2. Results and Discussion

### *2.1. Identification and Functional Characterization of* Ostreococcus *RCC809 Genes for "Front-End" Desaturases*

#### 2.1.1. Fatty Acid Composition of *Ostreococcus* RCC809

*Ostreococcus* RCC809 is the smallest known free-leaving marine picophytoeukaryote belonging to the "low-light" adapted ecotype of *Prasinophyceae* [13]. It is closely related to the two "high-light" adapted ecotypes of the genus *Ostreococcus*, *O. tauri* and *O. lucimarinus*, that have the ability to synthesize EPA and DHA via a series of alternating desaturation and elongation steps [12–16]. To explore the LC-PUFA pathway operating in *Ostreococcus* RCC809 we analyzed by GC-FID and GC-MS the fatty acid methyl esters (FAMEs) of total lipids from *Ostreococcus* RCC809 cultures growing at stationary phase. This analysis revealed the presence of several fatty acids belonging to the n-3 PUFA pathway (Figure 2). The most abundant fatty acid was 16:0 (20.1% of total fatty acids, TFA) followed by SDA (19.1% of TFA) and 16:1 (18.1% of TFA) (Supplementary Table S1). Compared to O. tauri, which contains on average 12% of DHA [13], the amount of this fatty acid in Ostreococcus RCC809 is rather low (1.83%); similarly, the levels of EPA are very low. The fatty acid content of Ostreococcus RCC809 is comparable to what has been found for O. lucimarinus, containing high levels of SDA (15%) and small amount of EPA and DHA [17]. Thus, the predominant omega-3 PUFA in Ostreococcus RCC809 is the Δ6-desaturated C18 fatty acid SDA, as opposed to either EPA or DHA.

**Figure 2.** Total fatty acid methyl esters of Ostreococcus RCC809. Fatty acid methyl esters (FAMEs) were prepared and analyzed by gas chromatography coupled with a flame ionization detector (GC-FID) as described in "Experimental Section", with peaks being identified by co-migration against known standards. The identity of major peaks is shown.



### 2.1.2. Identification and Functional Characterization in Yeast of a Putative Δ6-Desaturase from *Ostreococcus* RCC809

The genome of the green alga *Ostreococcus* RCC809 has been sequenced by US DOE Joint Genome Institute (JGI) and the predicted gene models are available for inspection and query [18]. We queried the predicted gene models via BLAST using previously characterized *N*-terminal cytochrome b5-fusion desaturases as query sequences. This analysis revealed the presence of several genes coding for putative omega-3 LC-PUFA desaturases. The top-scoring predicted open reading frames (ORFs) are listed in Table 1. Interestingly, no obvious Δ5-desaturase was identified by our searches, as might be expected for an organism that synthesizes EPA (Figure 1, Supplementary Table S1). The most obvious explanation for this absence is that the sequence of the *Ostreococcus* RCC809 genome is not yet complete and/or the genomic structure of the Δ5-desaturase is such that it has evaded the gene-prediction algorithms used to identify ORFs. The enzymatic activities of the two identified candidate desaturases (Table 1) were investigated by heterologous expression in *Saccharomyces cerevisiae*, and the deduced open reading frames were used as templates to chemically synthesize codon-optimized nucleotide sequences for expression in diatoms (based on the subsequent requirement to express the algal sequences in transgenic *Phaeodactylum tricornutum* and our observation that such diatom codon usage was readily accepted by *S. cerevisiae*). These synthetic coding sequences were cloned as *KpnI-SacI* fragments behind the galactose–inducible GAL1 promoter of the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA) and expressed in yeast in the presence of potential fatty acid substrates as previously described [7]. Total fatty acid methyl esters (FAMEs) from transgenic yeast were analyzed by GC-FID and the identity of novel peaks confirmed by GC-MS and co-migration with authentic standards.

**Table 1.** LC-PUFA biosynthetic genes cloned from *Ostreococcus* RCC809.

| Species | Protein ID | Amino Acids | Closest Match on Genbank, % Identity | Designation | Defined Function |
|---|---|---|---|---|---|
| *Ostreococcus* | 59992 | 461 | Δ6-desaturase from *O.lucimarinus* (82%) | **Ost809D6** | C18 Δ6-desaturase |
| RCC809 | | | Accession number: DAA34893.1 | | |
| *Ostreococcus* | 40461 | 459 | Δ4-desaturase from *O. lucimarinus* (85%) | **Ost809D4** | C22 Δ4-desaturase |
| RCC809 | | | Accession number: XP_001415743.1 | | |

Blast analysis using as a query the amino acid sequence of protein 59992 showed that the protein had high homology to previously reported acyl-CoA Δ6-desaturases from microalgae (Supplementary Figure S1). The protein from Ostreococcus RCC809 was most similar to the Δ6-desaturases from its closest relatives, O. lucimarinus and O. tauri which had an identity of 82% and 75%, respectively.

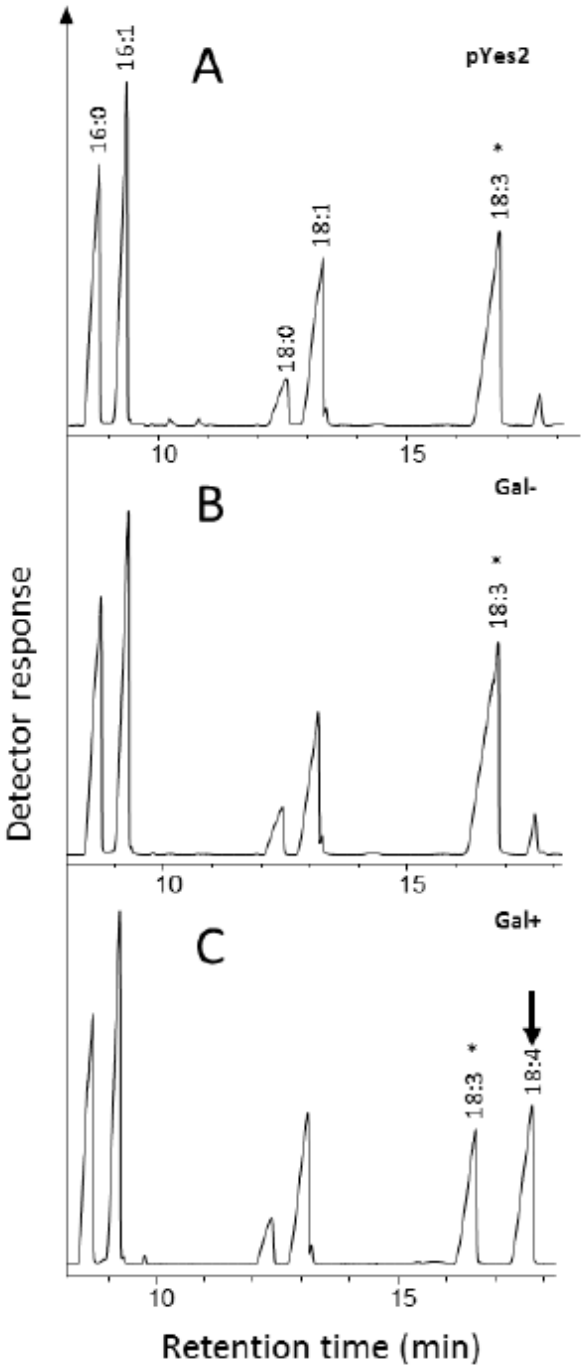
The substrate specifiity of the putative Δ6-desaturase was determined by exogenously supplying various substrate fatty acids in the growth media. As shown in Figure 3,

heterologous expression of the codon-optimized synthetic ORF encoding Ostreococcus RCC809 Protein 59992, predicted to encode a C18 Δ6-desaturase of 461 amino acids (aa), confirmed the enzymatic capability to convert exogenously supplied substrate α-linolenic acid (ALA; n-3) to the Δ6-desaturated product stearidonic acid (SDA; 18:4n-3). In the absence of galactose, the exogenous substrate ALA is not converted to SDA, since the transgene is not expressed. Thus, on the basis of these results, Ostreococcus RCC809 Protein 59992 was designated Ost809D6, and represents a new confirmed Δ6-desaturase member of the N-terminal cytochrome b5-fusion desaturase family. Ost809D6 displays high desaturation activity in yeast, converting about 54% of the available substrate ALA, with SDA accumulating to 18.5% of total fatty acids (Table 2). Ost809D6 only recognized the n-3 fatty acid ALA as a substrate, with the n-6 substrate LA showing no detectable desaturation in this heterologous yeast expression system. No activity was detected against exogenous 20:3n-6, 20:2n-6, 20:3n-3, 20:4n-3 and 22:5n-3. This strong preference for omega-3 versus omega-6 C18 substrates was also likely reflected in the fatty acid profile of Ostreococcus RCC809 (Supplementary Table S1), with accumulation of omega-6 (substrate) LA but not (product) GLA, and the inverse accumulation of omega-3 product (SDA) but not substrate (ALA). This strong omega-3 preference of Ost809D6 is distinct from a Δ6-desaturase with sequence-similarity identified from Ostreococcus tauri [14], which showed high activity towards both LA and ALA as substrates. It is more similar to Δ6-desaturase identified from M. squamata which accepts only ALA as a substrate [10]. Thus, Ost809D6 is potentially very useful for the exclusive production of Δ6-desaturated omega-3 fatty acids in transgenic plants.

**Table 2.** Fatty acid composition (%) of transgenic yeast. Values are the average of three independent experiments ± standard error. ND—not detected. The substrate supplemented to the culture is indicated in bold.

| | Construct | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fatty Acid Composition (Molar %) ± SD | | | | | | | |
| **Fatty Acid** | **Ost809D6 Gal −** | **Ost809D6 Gal +** | **Ost809D4 Gal −** | **Ost809D4 Gal +** | **FcElo6 Gal −** | **FcElo6 Gal +** | **FcElo6 Gal −** | **FcElo6 Gal +** |
| **16:0** | 22.3 ± 0.1 | 22.2 ± 0.4 | 32.8 ± 0.3 | 34.4 ± 0.2 | 24.5 ± 0.3 | 22.1 ± 0.1 | 20.1 ± 0.4 | 21.4 ± 0.1 |
| **16:1** | 25.4 ± 0.3 | 26.1 ± 0.3 | 38.9 ± 0.4 | 37.8 ± 0.3 | 26.8 ± 0.2 | 25.1 ± 0.3 | 22.3 ± 0.2 | 21.9 ± 0.3 |
| **18:0** | 3.7 ± 0.5 | 3.3 ± 0.3 | 7.9 ± 0.4 | 7.3 ± 0.5 | 3.7 ± 0.4 | 3.2 ± 0.3 | 3.7 ± 0.3 | 2.8 ± 0.4 |
| **18:1** | 13.4 ± 0.4 | 14.2 ± 0.4 | 17.8 ± 0.3 | 17.2 ± 0.4 | 15.3 ± 0.3 | 13.2 ± 0.5 | 3.4 ± 0.2 | 3.6 ± 0.3 |
| **LA** | ND | ND | ND | ND | ND | ND | ND | ND |
| **GLA** | ND | ND | ND | ND | **29.7 ± 0.2** | **22.3 ± 0.1** | **28.2 ± 0.4** | **18.6 ± 0.5** |
| **ALA** | **32.9 ± 0.2** | **15.7 ± 0.1** | ND | ND | ND | ND | ND | ND |
| **SDA** | 2.3 ± 0.6 | 18.5 ± 0.1 | ND | ND | ND | ND | **22.3 ± 0.3** | **12.2 ± 0.4** |
| **20:4*n*-3** | ND | ND | ND | ND | ND | ND | ND | 7.8 ± 0.3 |
| **DGLA** | ND | ND | ND | ND | ND | 14.1 ± 0.2 | ND | 11.7 ± 0.4 |
| **DPA** | ND | ND | **2.6 ± 0.3** | **2.8 ± 0.2** | ND | ND | ND | ND |
| **DHA** | ND | ND | ND | 0.5 ± 0.2 | ND | ND | ND | ND |

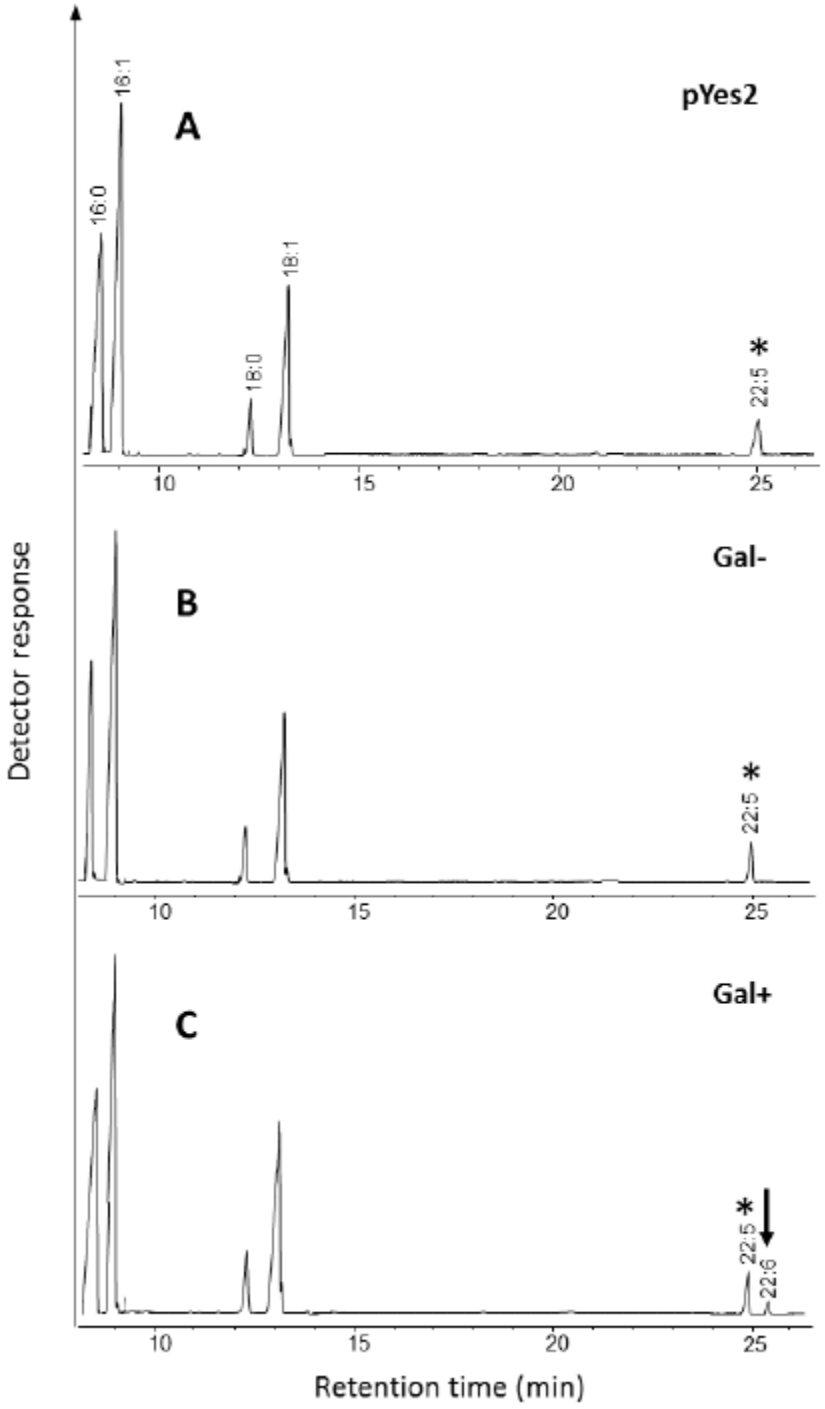
**Figure 3.** Functional characterization of *Ostreococcus* RCC809 C18 Δ6-desaturase. A synthetic gene encoding *Ostreococcus* RCC809 Protein 59992 was expressed in *S. cerevisiae* under the control of the galactose-inducible GAL promoter in the presence of exogenously supplied substrate α-linolenic acid (ALA) and galactose (**C**). The presence of the Δ6-desaturation product stearidonic acid (SDA) is indicated (arrowed). In the absence of galactose no conversion of ALA is seen (**B**). The profile of control yeast strain transformed with empty vector is also shown (**A**). The substrate supplemented to the cultures is indicated by an asterisk.



### 2.1.3. Identification and Functional Characterization of a Putative Δ4-Desaturase from *Ostreococcus* RCC809

Similar to as described above, the genome sequence of *Ostreococcus* RCC809 was searched with previously functionally characterized sequences of Δ4-desaturases and the presence of an ortholog (JGI protein ID # 40461) for a Δ4-desaturase was detected (Table 1). The deduced amino acid sequence was used as a query for similarity searches using BLAST analyses after which a multiple alignment was created (Supplementary Figure S2). The most similar proteins were Δ4-desaturase from *O. lucimarinus* and chloroplast Δ6-desaturase from Chlamydomonas which had the identities of 85% and 41%, respectively, although the protein demonstrated very low similarity to previously reported Δ4-desaturases [12,15,19,20]. The deduced open reading frame was again used as a template to chemically synthesise codon-optimized nucleotide sequences for expression in the diatom *P. tricornutum*. The synthetic ORF of the putative Δ4-desaturase was inserted as a *KpnI-SacI* fragment behind the galactose-inducible GAL1 promoter of the yeast expression vector pYES2 and was tested for activity against the appropriate 22:5*n*-3 (DPA) substrate (Figure 4). Expression of the synthetic predicted ORF encoding a polypeptide of 459 aa resulted in the Δ4-desaturation of DPA to DHA, with a conversion rate of 15.1% (0.5% accumulation of DHA, Table 2), confirming the function of this ORF as a C22 Δ4-desaturase and on this basis we designated this gene as Ost809D4. Note that in the absence of the inducer (galactose), no DHA is detected, nor in the absence of the Ost809D4 ORF. No activity was detected against exogenously supplied potential substrate for Δ5-desaturation, 20:3*n*-6 (DGLA) (data not shown). Thus, although *Ostreococcus* RCC809 synthesizes only limited levels of DHA, its genome encodes a fully functional enzyme for the terminal desaturation in the biosynthesis of DHA. In that respect, it maybe be that under some particular environmental conditions or lifecycle stages this gene is more actively expressed and higher levels of DHA are generated.

**Figure 4.** Functional characterization of *Ostreococcus* RCC809 C22 Δ4-desaturase. In the presence of exogenously supplied substrate (22:5*n*-3) and galactose, the accumulation of the Δ4-desaturation product DHA is detected (**C**) when total yeast fatty acids are analyzed. In the absence of galactose, no conversion of 22:5*n*-3 is seen (**B**). The profile of control yeast strain transformed with empty vector is also shown (**A**). The substrate supplemented to the cultures is indicated by an asterisk.

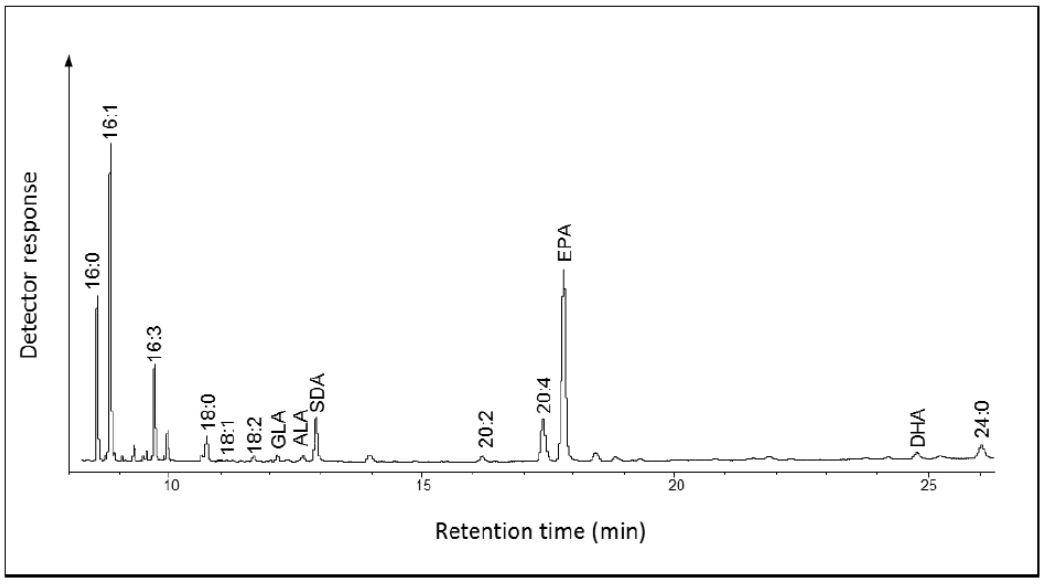


## *2.2. Identification and Functional Characterization of a Putative Δ6-Elongase from* Fragilariopsis cylindrus

### 2.2.1. Fatty Acid Composition of *Fragilariopsis cylindrus*

The GC-FID analysis of FAMEs of total lipids from stationary phase cultures of *Fragilariopsis cylindrus* (*F. cylindrus*) (Figure 5) revealed that the most abundant fatty acid in this diatom was EPA (31.4% of TFA) followed by 16:1 and 16:0 (24.5% and 12.1% respectively) (Supplementary Table S1). Similar to *Ostreococcus* RCC809, only low levels of DHA were observed, although SDA levels were markedly lower in *F. cylindrus*. On the basis of significant levels of the C20 fatty acid EPA, it was indicative that this diatom contains a Δ6-elongase activity capable of elongating SDA to ETA prior to Δ5-desaturation, and for this reason, efforts were focused on identifying this gene.

**Figure 5.** Total fatty acid methyl esters of *F. cylindrus*. FAMEs were prepared and analyzed by GC-FID as described in "Experimental Section", with peaks being identified by co-migration against known standards. The identity of major peaks is shown.

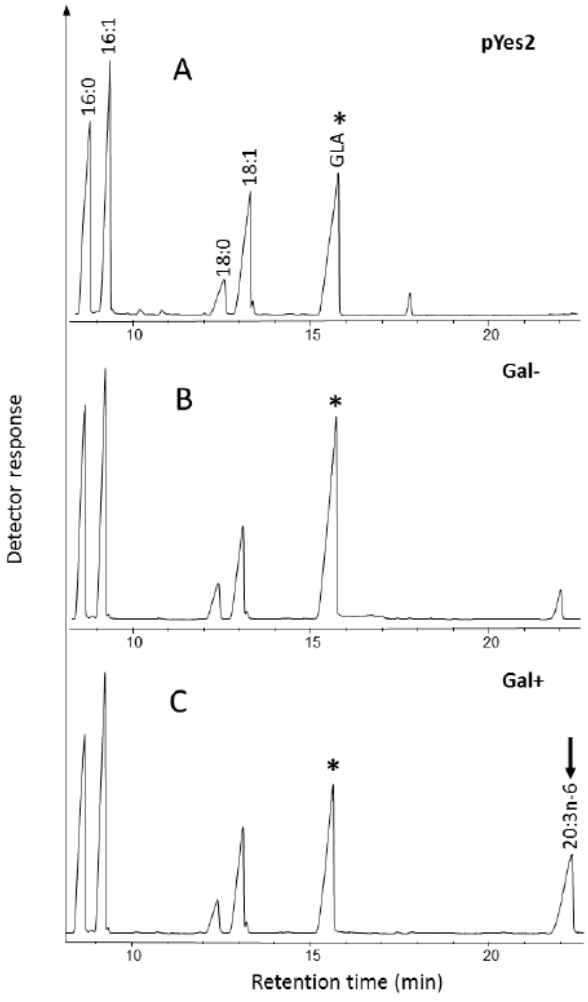



### 2.2.2. Functional Expression in Yeast of a Putative Elongase

Similar to the approach used for *Ostreococcus* RCC809, the publicly available genome sequence of the marine diatom *F. cylindrus* [21] was probed via BLAST search interface, using previously characterized Δ6-elongase sequences as the query template. This identified one strong candidate, and the deduced ORF of 287 aa (designated Frag#177742) was used as a query for similarity searches. Frag#177742 showed highest identity (70%) to Δ6-elongases from diatoms, *Thalassiosira pseudonana* [15] and *Phaeodactylum tricornutum* (Accession number AAW70157) (Supplementary Figure S3). To confirm the function of this putative

elongase sequence, the synthetic ORF was expressed in yeast in the presence of exogenous 18:3*n*-6, GLA (Figure 6). Expression of Frag#177742 in yeast demonstrated that this sequence directed the elongation of GLA to generate 20:3*n*-6, DGLA (14.1% of TFA, 38.1% conversion rate). Thus, Frag#177742 was redesignated FcElo6, on the basis of possessing *bona fide* elongating activity specific for C18 Δ6-unsaturated substrates. The substrate specificity of FcElo6 was analyzed by exogenously supplying equal quantities of GLA and SDA in the growth media. As shown in Table 2, FcElo6 elongated SDA to generate 20:4*n*-3 at similarly high proportions (39% conversion rate). No elongation was observed with other exogenous potential substrates tested (LA and EPA, data not shown).

**Figure 6.** Functional characterization of *F. cylindrus* C18 Δ6-elongase. A synthetic gene encoding *F. cylindrus* Protein 177742 was expressed in *S. cerevisiae* under the control of the galactose-inducible GAL promoter. In the presence of exogenously supplied substrate (GLA) and galactose, the presence of the elongation product 20:3*n*-6 is detected when total yeast fatty acids are analyzed (**C**). In the absence of galactose, no elongation of GLA is seen (**B**). The profile of control yeast strain transformed with empty vector is also shown (**A**). The substrate supplemented to the cultures is indicated by an asterisk. Arrow indicates additional peak corresponding to DGLA, 20:3*n*-6.



## 3. Experimental Section

### *3.1. Growth and Harvesting of Microalgal Strains*

Cultures of *Ostreococcus* RCC809 were grown in ESAW medium [22,23] at 20 °C under 20 μM photons $m^{-2}$ $s^{-1}$. Cultures were agitated manually every two days to prevent aggregation. Cells were collected by centrifugation.
Cultures of *F. cylindrus* were grown in Aquil media [24–26] at 4 °C under illumination at 40 μM photons $m^{-2}$ $s^{-1}$, and were shaken manually from time to time. Cells were collected by centrifugation.

### *3.2. Identification and Cloning of Putative PUFA Genes*

Genomes of the unicellular algae *Ostreococcus* RCC809 and the marine diatom *F. cylindrus* were analyzed with BLAST using *N*-terminal cytochrome b5-fusion desaturases and ELO-like elongating activity as templates. This analysis revealed the presence of several genes coding for putative PUFA desaturases and an elongase. The putative Δ6- and Δ4-desaturase sequences from *Ostreococcus* RCC809 and Δ6 elongase sequence from *F. cylindrus*, were used as templates to chemically synthesize (Genscript Corporation, Piscataway, NJ, USA) codon-optimized nucleotide sequences for expression in diatoms. The codon-optimized genes were subcloned into the *KpnI-SacI* sites present in the galactose inducible yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA).

### *3.3. Functional Expression in Yeast*

ORFs encoding putative desaturation and elongation activities were introduced in *S. cerevisiae* strain W303-1A by a lithium acetate method. Cultures were grown on synthetic dextrose minimal medium minus uracil at 22 °C in the presence of 2% (w/v) raffinose for 48 h and expression of the transgenes was induced by the addition of galactose to 2% (w/v) in the presence of 0.25 mM of the corresponding fatty acid and 1% (w/v) tergitol-Nonidet P-40 (Sigma-Aldricht, Haverhill, UK) as described [8].

### *3.4. Fatty Acid Analysis*

Fatty acids were extracted and methylated as described previously [27]. Total fatty acids were analyzed by gas chromatography coupled with a flame ionization detector (GC-FID) of methyl ester derivatives. FAME samples were analyzed by as liquid chromatography using a Hewlett-Packard 6890 series Gas Chromatograph and an Alltech AT-225 (30 m × 0.32 mm × 0.3 μm) capillary column. Inlet and detector temperature was set to 250 °C and 1 μL of each sample was analyzed using splitless injection and a constant flow rate of 2 mL/min. The oven temperature cycle was set a follows: A start temperature of 50 °C was held for 1 min to allow vaporized samples and the solvent (hexane) to condensate at the front of the column. Oven temperature was then increased rapidly to 190 °C at a rate of 40 °C/min followed by a slower increase to 220° C at a rate of 1.5 °C/min. The final temperature of 220 °C was held for 1 min

giving a total run time of 25 min 50 s per sample. FAMEs were detected using a Flame Ionization Detector (FID). Chromatograms were analysed using the Agilent ChemStation software Rev B.04.02 (118). Peak area percentages (area %) were converted to molecular percentages (mole %) to correct the error inherent to FID due to the different carbon number of each compound.

## 4. Conclusions

In conclusion, through a database search of the genomes of two primary producers of LC-PUFAs, we have identified and functionally characterized three novel genes involved in the biosynthesis of the nutritionally important omega-3 polyunsaturated fatty acids in marine microalgae. Of particular interest is the Δ6-desaturase from the unicellular photoautotrophic green alga *Ostreococcus* RCC809, which demonstrated activity with specificity towards omega-3 substrates, making this an interesting candidate for heterologous expression in transgenic plants. Previous studies by us [7,8] have identified unusual higher plant Δ6-desaturases from the *Primulaceae* which showed distinct preferences for either omega-3 or omega-6 C18 substrates. Similar to this present study, it was not possible to identify the precise amino acid determinants of such specificities, despite a high degree of similarity between enzymes with different substrate preferences. The *Ostreococcus* RCC809 activity described here is another example of an algal desaturase with strong selectivity for *n*-3 substrates and better conversion rates of the *n*-3 substarte ALA than for previously reported Δ6-desaturases from microalgae [10–12]. One additional factor which remains to be determined for this particular enzyme is the nature of substrate used by the desaturase. One of the most closely related orthologs of Ost809D6 is the Δ6-desaturase from *Ostreococcus tauri* (Supplementary Figure S2) which has been shown to prefer acyl-CoA substrates as opposed to the phospholipid-linked substrates more normally associated with lower eukaryotic desaturation [14]. Based on our present studies, it is not possible to infer any such tendency in the *Ostreococcus* RCC809 desaturase, but the definitive experiments remain to be carried out. Collectively, the algal genes functionally characterized in this study further add to our understanding of the biosynthesis of the vital and valuable omega-3 LC-PUFAs in the marine food-web, and also provide additional molecular tools with which to attempt the heterologous reconstruction of that biosynthetic pathway in transgenic hosts such as plants.

## Acknowledgments

Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council (BBSRC), UK. RV is the recipient of a BBSRC studentship funded through the IBTI Club. The authors would like to thank Jan Strauss at the University of East Anglia for providing cell pellets of *F. cylindrus* for lipid analysis.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Voigt, R.G.; Jensen, C.L.; Fraley, J.K.; Rozelle, J.C.; Brown, F.R.; Heird, W.C. Relationship between omega-3 long-chain polyunsaturated fatty acid status during early infancy and neurodevelopmental status at 1 year of age. *J. Hum. Nutr. Diet* **2000**, *15*, 111–120.

2. Calder, P.C. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. Lipids 2003, 38, 343–352.

3. Haslam, R.P.; Ruiz-Lopez, N.; Eastmond, P.; Moloney, M.; Sayanova, O.; Napier, J.A. The modification of plant oil composition via metabolic engineering—better nutrition by design. Plant Biotechnol. J. 2013, 11, 157–168.

4. Ruiz-Lopez, N.; Haslam, R.P.; Usher, S.L.; Napier, J.A.; Sayanova, O. Reconstitution of EPA and DHA biosynthesis in Arabidopsis: Iterative metabolic engineering for the synthesis of n-3 LC-PUFAs in transgenic plants. Metab. Eng. 2013, 17, 30–41.

5. Petrie, J.R.; Shrestha, P.; Zhou, X.R.; Mansour, M.P.; Liu, Q.; Belide, S.; Nichols, P.D.; Singh, S.P. Metabolic Engineering Plant Seeds with Fish Oil-Like Levels of DHA. PLoS One 2012, 7, e49165.

6. Venegas-Calerón, M.; Sayanova, O.; Napier, J.A. An alternative to fish oils: Metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. Prog. Lipid Res. 2010, 49, 108–119.

7. Sayanova, O.V.; Beaudoin, F.; Michaelson, L.V.; Shewry, P.R.; Napier, J.A. Identification of Primula fatty acid Δ6-desaturases with n-3 substrate preferences. FEBS Lett. 2003, 542, 100–104.

8. Sayanova, O.; Haslam, R.; Venegas-Calerón, M.; Napier, J.A. Identification of Primula "front-end" desaturases with distinct n-6 or n-3 substrate preferences. Planta 2006, 224, 1269–1277.

9. García-Maroto, F.; Mañas-Fernández, A.; Garrido-Cárdenas, J.A.; Alonso, D.L. Substrate specificity of acyl-Δ6-desaturases from Continental versus Macaronesian Echium species. Phytochemistry 2006, 67, 540–544.

10. Hoffmann, M.; Wagner, M.; Abbadi, A.; Fulda, M.; Feussner, I. Metabolic engineering of omega3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. J. Biol. Chem. 2008, 283, 22352–22362.

11. Petrie, J.R.; Shrestha, P.; Mansour, M.P.; Nichols, P.D.; Liu, Q.; Singh, S.P. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Delta6-desaturase with omega-3 preference from the marine microalga Micromonas pusilla. Metab. Eng. 2010, 12, 233–240.

12. Petrie, J.R.; Liu, Q.; Mackenzie, A.; Shrestha, P.; Mansour, M.P.; Robert, S.S.; Frampton, D.F.; Blackburn, S.I.; Nichols, P.D.; Singh, S.P. Isolation and characterization of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. Mar. Biotechnol. 2010, 12, 430–438.

13. Demir-Hilton, E.; Sudek, S.; Cuvelier, M.L.; Gentemann, C.L.; Zehr, J.P.; Worden, Z. Global distribution patterns of distinct clades of the photosynthetic picoeukaryote Ostreococcus. ISME J. 2011, 5, 1095–1107.

14. Domergue, F.; Abbadi, A.; Zähringer, U.; Moreau, H.; Heinz, E. In vivo characterization of the first acyl-CoA Delta6-desaturase from a member of the plant kingdom, the microalga Ostreococcus tauri. Biochem. J. 2005, 389, 483–490.

15. Meyer, A.; Kirsch, H.; Domergue, F.; Abbadi, A.; Sperling, P.; Bauer, J.; Cirpus, P.; Zank, T.K.; Moreau, H.; Roscoe, T.J.; et al. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. J. Lipid Res. 2004, 45, 1899–1909.

16. Ahman, K.; Heilmann, M.; Feussner, I. Identification of a Δ4-desaturase from microalga Ostreococcus lucimarinus. Eur. J. Lipid Sci. Technol. 2011, 113, 832–840.

17. Wagner, M.; Hoppe, K.; Czabany, T.; Heilmann, M.; Daum, G.; Feussner, I.; Fulda, M. Identification and characterization of an acyl-CoA: Diacylglycerol acyltransferase 2 (DGAT2) gene from the microalga O. tauri. Plant Phys. Biochem. 2010, 48, 407–416.

18. Joint Genome Institute Ostreococcus sp. RCC809 2.0 genome project. Available online: http://genome.jgi-psf.org/OstRCC809_2/OstRCC809_2.home.html (accessed on 5 April 2012).

19. Tonon, T.; Sayanova, O.; Michaelson, L.V.; Qing, R.; Harvey, D.; Larson, T.R.; Li, Y.; Napier, J.A.; Graham, I.A. Fatty acid desaturases from the microalga Thalassiosira pseudonana. FEBS J. 2005, 272, 3401–3412.

20. Sayanova, O.; Haslam, R.; Venegas-Calerón, M.; Ruiz-Lopez, N.; Worthy, C.; Rooks, P.; Allen, M.J.; Napier, J.A. Identification and functional characterization of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore Emiliania huxleyi. Phytochemistry 2011, 72, 594–600.

21. Joint Genome Institute Fragilariopsis cylindrus genome project. Available online: http://genome.jgi-psf.org/Fracy1/Fracy1.home.html (accessed on 10 May 2012).
22. Harrison, P.J.; Waters, R.E.; Taylor, F.J.R. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. J. Phycol. 1980, 16, 28–35.

23. Berges, J.A.; Franklin, D.J.; Harrison, P.J. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the past two decades. J. Phycol. 2001, 37, 1138–1145.

24. Morel, F.M.M.; Rueter, J.G.; Anderson, D.M.; Guillard, R.R.L. Aquil: A chemically defined phytoplankton culture medium for trace metal studies. J. Phycol. 1979, 15, 135–141.

25. Price, N.M.; Harrison, G.I.; Hering, J.G.; Hudson, R.J.; Nirel, P.M.V.; Palenik, B.; Morel, F.M.M. Preparation and chemistry of the artificial algal culture medium Aquil. Biol. Oceanogr. 1989, 6, 443–461.

26. Sunda, W.G.; Price, N.M.; Morel, F.M.M. Trace metal ion buffers and their use in culture studies. In Algal Culturing Techniques; Anderson, R.A., Ed.; Academic Press: Amsterdam, The Netherlands, 2005; pp. 35–63.

27. Sayanova, O.; Smith, M.A.; Lapinskas, P.; Stobart, A.K.; Dobson, G.; Christie, W.W.; Shewry, P.R.; Napier, J.A. Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of delta6-desaturated fatty acids in transgenic tobacco. Proc. Natl. Acad. Sci. USA 1997, 94, 4211–4216.

## APPENDIX II) SUPPLEMENTARY INFORMATION FOR APPENDIX I), PUBLISHED IN THE JOURNAL MARINE DRUGS (VAEZI ET AL, 2013)

# Supplementary Information

**Table S1.** Fatty acid composition of *Ostreococcus RCC809* (*O. RCC809*) and *F. cylindrus* cultures in stationary phase. Values are the average of three independent experiments (±standard deviation). ND—not detected.

**Figure S1.** Multiple sequence alignment of Ost809D6 with Δ6-desaturases from *Mantoniella squamata* (*M.squaD6*, CAQ30479.1) [1], *Micromonas pusilla* (*M.pusD6*, XP_002502445.1) [2], *Ostreococcus lucimarinus* (*O.lucD6*, DAA34893.1) [3] and *Ostreococcus tauri* (*O.tauriD6*, XP_003082578.1) [4]. Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of conserved cytochrome $b_5$ domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W.

**Figure S2.** Multiple sequence alignment of Ost809D4 with Δ4-desaturases from *Pavlova lutheri* (*P.lutD4*, AAQ98793.1) and *Pavlova salina* (*P.salD4*, AY926606.1) [4], *Isochrysis galbana* (*I.galD4*, AY630574) [5], *Ostreococcus lucimarinus* (*O.lucD4*, XM_001415706.1) [6], and *Emiliania huxleyi* (*E.huxD4*, [7]). Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of conserved cytochrome $b_5$ domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W. A related Δ4-desaturase from *Thalassiosira pseudonana* (TpD4, AAX14506.1) [8] was omitted from this line-up on the basis of poor alignment.

**Figure S3.** Multiple sequence alignment of FcElo6 with Δ6-elongasse from *Thalassiosira pseudonana* (TpElo6, AY591337.1) and *Ostreococcus tauri* (OtElo6, AY591335) [5]. Conserved amino acid residues are indicated with an asterisk, whereas conserved motifs are framed. The alignment was obtained using CLUSTAL W.

**Figure S4.** Codon-optimised nucleotide sequences. (**A**), Ost 809D6; (**B**), Ost809D4; (**C**) Fc ELO6.

**Table S1.** Fatty acid composition of *Ostreococcus RCC809* (*O. RCC809*) and *F. cylindrus* cultures in stationary phase. Values are the average of three independent experiments (±standard deviation). ND—not detected.

| | Composition (Molar %) ± SD | |
|---|---|---|
| **Fatty Acid** | ***O. RCC809*** | ***F. cylindrus*** |
| **16:0** | 20.12 ± 0.6 | 12.1 ± 0.2 |
| **16:1*n*-7** | 18.1 ± 0.3 | 24.5 ± 0.1 |
| **16:4*n*-3** | 5.08 ± 0.4 | ND |
| **18:0** | 1.24 ± 0.5 | 2.4 ± 0.3 |
| **18:1*n*-9** | 8.17 ± 0.2 | ND |
| **18:1*n*-7** | 8.03 ± 0.1 | ND |
| **LA** | 9.12 ± 0.2 | 0.7 ± 0.3 |
| **GLA** | 3.29 ± 0.3 | 1 ± 0.4 |
| **ALA** | 3.98 ± 0.4 | 0.8 ± 0.3 |
| **SDA** | 19.13 ± 0.6 | 5.7 ± 0.5 |
| **20:2*n*-6** | 0.65 ± 0.4 | 1 ± 0.2 |
| **20:4*n*-3** | ND | 7.4 ± 0.1 |
| **EPA** | 1.26 ± 0.4 | 31.4 ± 0.3 |
| **DHA** | 1.83 ± 0.5 | 2.5 ± 0.1 |
| **24:0** | ND | 1.4 ± 0.2 |

**Figure S1.** Multiple sequence alignment of Ost809D6 with Δ6-desaturases from *Mantoniella squamata* (*M.squaD6*, CAQ30479.1) [1], *Micromonas pusilla* (*M.pusD6*, XP_002502445.1) [2], *Ostreococcus lucimarinus* (*O.lucD6*, DAA34893.1) [3] and *Ostreococcus tauri* (*O.tauriD6*, XP_003082578.1) [4]. Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of conserved cytochrome $b_5$ domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W.

```
Ost809D6        ------------------MRVETEDDNVPTVTVGLSEESDGMKGARNPGARAWKSTLEP
O.lucD6         MCVETTEGTSRTMANERTSSSSSLSEGGTPTVTVGMGSE-DAGKKTRNASVTAWTKELEP
O.tauriD6       ------------------MCVETENNDGIPTVEIAFDGE----RERAEANVKLSAEKMEP
M.squaD6        ----------------------------MCPPKESTRKNAGGPLTRGKLSADLAKLEP
M.pusD6         ----------------------------------------MTRGNKAKLDNSKLAKLEP
                                                                  :**

                                            ___________
Ost809D6        HAVAKSFDRRWVKVDGVEYDVTDFKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKA
O.lucD6         HAIAKTFERRYVTIEGVEYDVTDFKHPGGSVIYYMLSNTGADATEAFKEFHYRSKKARKA
O.tauriD6       AALAKTFARRYVVIEGVEYDVTDFKHPGGTVIFYALSNTGADATEAFKEFHHRSRKARKA
M.squaD6        HKLAQTFDTRWVRVGDVEYDVTNFKHPGGSVIFYMLSNTGADATEAFNEFHMRSPKAWKM
M.pusD6         HKLAQTFEQRWVRIDDVEYDVTNFKHPGGSVIFYMLSNTGADATEAFKEFHMRSPKAWKM
                  :*::*  *:* : .******:******:**:* ************:*** ** ** *

Ost809D6        LAALPQREPEDAS--PVEDANMLKDFAKWRKDLEREGFFKPSPAHVAYRFAELAAMFALG
O.lucD6         LAALPHKPVDAATREPIEDEAMLKDFAQWRKELEREGFFKPSPAHVAYRFAELAAMFALG
O.tauriD6       LAALPSRP---AKTAKVDDAEMLQDFAKWRKELERDGFFKPSPAHVAYRFAELAAMYALG
M.squaD6        LKALPNRPAETPRSQ-DPDGPMLEDFAKWRAQLEKEGFFKPSIAHVAYRIAELAAMFALG
M.pusD6         LKALPQRPAETPRSA-DPDAPMLQDFARWRAELEKEGFFEPSRLHLAYRCLELCATFALG
                * *** :    .      *  **:***:** :**::***:**  *:***  **.* :***

Ost809D6        TALMYARWHATSVFVTACFFGARCGWVQHEGGHSSLTGSIWWDKRIQAFTAGFGLASSGD
O.lucD6         TALMHARWHVASVIVYSCFFGARCGWVQHEGGHNSLTGNIWWDKRIQAFAAGFGLASSGD
O.tauriD6       TYLMYARYVVSSVLVYACFFGARCGWVQHEGGHSSLTGNIWWDKRIQAFTAGFGLAGSGD
M.squaD6        CYIMSLGYPVVASIVFGAFFGARCGWVQHEGGHNSLTGNIWLDKRIQAATCGFGLSTSGD
M.pusD6         TFLMYIGRPLLASIVYGAFFGARCGWVQHEGGHNSLTGSIWWDKRIQAATCGFGLSTSGD
                  :*       : :* ..***************.****.** ****** :.****: ***

Ost809D6        MWNLMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEENRPRKFSKLWLRVQAWTFVPVTS
O.lucD6         MWNNMHNKHHATPQKVRHDMDLDTTPTVAFFNSAVEENRPRGFSKLWLRLQAWTFVPVTS
O.tauriD6       MWNSMHNKHHATPQKVRHDMDLDTTPAVAFFNTAVEDNRPRGFSKYWLRLQAWTFIPVTS
M.squaD6        MWNQMHNKHHATPQKVRHDMDLDTTPAVAFFKTAVEDNRPRGFSRAWSRAQAWTFVPVTS
M.pusD6         MWNQMHNKHHATPQKVRHDMDLDTTPAVAFFDTAVEDNRPRGFSKTWARAQAWTFVPITS
                *** ***********************:****.:***:**** **: * * *****:*:**

Ost809D6        -GLVLLAWMYLLHPRHIARRKNYEEAAWIVAAHVIRTSVIKAVTGYSWITCYGLFLSTMW
O.lucD6         -GMVLFFWMFVLHPRNALRRKSFEEAAWMFSAHVIRTAVIKAVTGYSWIASYGLFAATMW
O.tauriD6       -GLVLLFWMFFLHPSKALKGGKYEELVWMLAAHVIRTWTIKAVTGFTAMQSYGLFLATSW
M.squaD6        GLLVQMFWIYVLHPRQVARKKNYEEASWMILSHVLRTATIKYAGGYSWPVAYLWFSFGNW
M.pusD6         GVLVQMFWIYVLHPRQVLRKKNYEEASWMFLSHVVRTAVIKLALGCGTAEAYGWFWVGNW
                  :* : *::.*** :  :  .:**  *:. :**:** .** . *     .*  *    *

Ost809D6        VSGCYLFAHFSTSHTHLDVVPSDKHLSWVRYAVDHTIDIDPSKSVVNWLMGYLNCQVIHH
O.lucD6         ASGCYLFAHFSTSHTHLDVVPSDKHLSWVRYAVDHTIDINPNNSVVNWLMGYLNCQVIHH
O.tauriD6       VSGCYLFAHFSTSHTHLDVVPADEHLSWVRYAVDHTIDIDPSQGWVNWLMGYLNCQVIHH
M.squaD6        IAYMYLFAHFSTSHTHLEVVPSDKHISWVNYAVDHTVDIDPSKGYVNWLMGYLNCQVIHH
M.pusD6         IAYMYLFAHFSTSHTHLDVVPSDKHISWVNYAVDHTVDINPRNSIVNWLMGYLNCQVIHH
                 :  ************:***:*:*:***.******:**:* :. ***************

Ost809D6        LFPDMPQFRQPEVSRRFVSFAKKWNLNYKVMSYYGAWKATFGNLNEVGKHYYIQGSQITK
O.lucD6         LFPDMPQFRQPEVSRRFVPFAKKWNLNYKVLTYYGAWKATFGNLNDVGKHYYVHGSQRVK
O.tauriD6       LFPSMPQFRQPEVSRRFVAFAKKWNLNYKVMTYAGAWKATLGNLDNVGKHYYVHGQHSGK
M.squaD6        LFPDMPQFRQPEVSRRFVAFAKKWNLNYKVLTYYGAWKATFTNLDTVGQHYYKHGKAHAH
M.pusD6         LFPDMPQFRQPEVSRRFVAFAEKWNLNYKVLTYYGAWKATFSNLDRVGQHYYVNGKAKAH
                ***.***************.**:*********::* ******: **: **:*** :*.   :
```

**Figure S2.** Multiple sequence alignment of Ost809D4 with Δ4-desaturases from *Pavlova lutheri* (*P.lutD4*, AAQ98793.1) and *Pavlova salina* (*P.salD4*, AY926606.1) [4], *Isochrysis galbana* (*I.galD4*, AY630574) [5], *Ostreococcus lucimarinus* (*O.lucD4*, XM_001415706.1) [6] and *Emiliania huxleyi* (*E.huxD4*, [7]). Conserved histidine boxes are shaded in grey. Conserved amino acid residues are indicated with an asterisk. The position of conserved cytochrome $b_5$ domain motif is marked with a solid line. The alignment was obtained using CLUSTAL W. A related Δ4-desaturase from *Thalassiosira pseudonana* (TpD4, AAX14506.1) [8] was omitted from this line-up on the basis of poor alignment.

```
Ost809D4        ---MPTTRSRARVTTPPRETPTRANTVAALDPERKYTRIRGVVYDVTDFASRHPGGAQLL
O.lucD4         --MPSAARSRASKRANATTDVATTAPEATLDPTRAYTRYRGVVYDVTEFQHRHPGGAQLL
P.lutD4         MPPSAASEGGVAELRAAEVAS---YTRKAVDERPDLTIVGDAVYDAKAFRDEHPGGAHFV
P.salD4         MPPSAAKQMGASTGVHAGVTDSSAFTRKDVADRPDLTIVGDSVYDAKAFRSEHPGGAHFV
I.galD4         -------------MCNAAQVETQALRAKEAAKPTWTKIHGRTVDVETFR--HPGGN-IL
EhuxD4          ----------------------MGGAGASEAERPKWTTIHGRHVDVSKFR--HPGGN-II
                                                *   .   *.  *   **** ::

Ost809D4        SLCVGRDATILVESHHLRPEVVQKYLKTLPVVEGAAGAFGPEETFPKPLDSDLYRKIQGR
O.lucD4         SLCVGRDATILIESHHLRPEVVRKYMKTLPVVEGAAGAFGKEETFPKPLDSAVYRAIQRR
P.lutD4         SLFGGRDATEAFMEYHRRAWPKARMSKFFVGSLDASEKPTQADSAYLRLCAEVNALLPKG
P.salD4         SLFGGRDATEAFMEYHRRAWPKSRMSRFHVGSLASTEEPVAADEGYLQLCARIAKMVPSV
I.galD4         DLFLGMDATTAFETFHGHHKGAWKMLKTLPEKEVAAADIPAQKEEHVAEMTRLMASWRER
EhuxD4          ELFYGMDSTSAFEQFHGHHKGAWKMLKALPTKEVDPADVPQQPQEHVAEMTRLMTSWRER
                .*  * *:*  .  .* :    :  :       .             : :

Ost809D4        VRKEIVEPLKMTRGREPHGRGWCVLDAGVVLAFFAFALGVYWKTPTVATGCLLGLAGYWS
O.lucD4         VRDEVVEPMKAKSGREAHGRGGCVVDAGVVVLTFVAAMVAYWRAPSALTGCALGLAGYWS
P.lutD4         SGGFAPPSYWLKAA------------ALVVAAVSIEGYMLLRGKTLLLSVFLGLVFAWI
P.salD4         SSGFAPASYWVKAG------------LILGSAIALEAYMLYAGKRLLPSIVLGWLFALI
I.galD4         GLFKPRPVASSIYG------------LCVIFAIAASVACAPYAPVLAGIAVGTCWAQC
EhuxD4          GLFKPRPVASGIYG------------LAVVAAIVACIACAPHAPVLSGIGLGSCWAQC
                             .             :                  .  :*

Ost809D4        GTGLQHTANHGGLAKSGFWNQFWGWLGNDVAIGKSSVEWRYHHMVSHHSYCNDADLDQDV
O.lucD4         GTGLQHTANHGGLAKSGFWNQFWGWLGNDVAIGKSSVEWRYHHMVSHHSYCNDADLDQDV
P.lutD4         GLNIQHDANHGALSRHSVINYCLGYA--QDWIGGNMVLWLQEHVVMHHLHTNDVDADPDQ
P.salD4         GLNIQHDANHGALSKSASVNLALGLC--QDWIGGSMILWLQEHVVMHHLHTNDVDKDPDQ
I.galD4         G-FLQHMGGHREWGRTWSFAFQHLFEG---LLKGGSASWWRNRHNKHHAKTNVLGEDGD-
EhuxD4          G-FLQHMGGHREWGVRYSFLLQHFFEG---LLKGGSASWWRNRHNKHHAKTNVLGEDGD-
                *  :** ..*   .                  :  .   *  .:   **   *  . * *

Ost809D4        YTALPLLRLDPSQELKWFHRYQAFYAPLMWPMLWLAAQFGDAQNILVDKASPGVEYKGLM
O.lucD4         YTALPLLRLDPSQELKWFHRYQAFYAPLMWPFLWLAAQVGDAQNILIDRASPGVEYKGLM
P.lutD4         K-AHGVLRLKPTDGWMPWHALQQLYILPGEAMYAFKLLFLDALELLAWRWE-GEKISPLA
P.salD4         K-AHGALRLKPTDAWSPMHWLQHLYLLPGETMYAFKLLFLDISELVMWRWE-GEPISKLA
I.galD4         ------LRTTPFFAWDP----TLAKKVPDWSLRTQAFTFLPALGAYVFVFAFTVRKYSVV
EhuxD4          ------LRTTPFFAWDP----TLAKKVPDWSLKTQAFTFLPALGAYVFVFAFTIRKYAVV
                      **  *                  .:     .                   :

Ost809D4        KLEVALYVLGKFLHFSLLLGVPAYLHGFANAIVPFIAYGAFGSFVLCWFFIVSHNLEALT
O.lucD4         KNEIALYLLGKVLHFGLLLGVPAYIHGLSNVIVPFLAYGAFGSFVLCWFFIVSHNLEALT
P.lutD4         RALFAPAVACKLGFWARFVALPLWLQPTVHTALCICATVCTGSFYLAFFFFISHNFDGVG
P.salD4         GYLFMPSLLLKLTFWARFVALPLYLAPSVHTAVCIAATVMTGSFYLAFFFFISHNFEGVA
I.galD4         KRLWHEVALMVAHYALFSWALSAAGASLSSGLTFYCTGYAWQGIYLGFFFGLSHFAVERV
EhuxD4          KKLWHELALMIAHYAMFYYALQLAGASLGSGLAFYCTGYAWQGIYLGFFFGLSHFAVERV
                      .    .:             :     .: * :** :**

Ost809D4        PIN-----LSKSTKNDWGAWQIETSASWGN--GFWSFFSGGLNLQIEHHLFPGCAHNLYP
O.lucD4         PMN-----LSKSTKNDWGAWQIETSASWGN--SFWSFFSGGLNLQIEHHLFPGCAHNLYP
P.lutD4         SVGPKG---SLPRSATFVQRQVETSSNVGG--YWLGVLNGGLNFQIEHHLFPRLHHSYYA
P.salD4         SVGPDGSITSMTRGASFLKRQAETSSNVGG--PLLATLNGGLNYQIEHHLFPRVHHGFYP
I.galD4         PS----------TATWLESTMMGTVDWGGSSAFCGYLSGFLNIQIEHHMAPQMPMENLR
EhuxD4          PS----------TATWLESSMIGTVDWGGSSAFCGYVSGFLNIQIEHHMAPQMPMENLR
                .             :      : . *.     . ..* ** *****: *
```

**Figure S3.** Multiple sequence alignment of FcElo6 with Δ6-elongasse from *Thalassiosira pseudonana* (TpElo6, AY591337.1) and *Ostreococcus tauri* (OtElo6, AY591335) [5]. Conserved amino acid residues are indicated with an asterisk, whereas conserved motifs are framed. The alignment was obtained using CLUSTAL W.

```
FcElo6          ----------------------MDEYKATLESVGDAIIQWADPESQFTGFTKGWFLTDFTS
TpElo6          ----------------------MDAYNAAMDKIGAAIIDWSDPDGKFRADREDWWLCDFRS
OtElo6          MSGLRAPNFLHRFWTKWDYAISKVVFTCADSFQWDIGPVSSSTAHLPAIESPTPLVTSLL
                                      .   :  :..   *   :.. .:: .  .   *

FcElo6          AFSIALVYVLFVIIGSQVMKVLPAIDPYPIKFFYNVSQIMLCAYMTIEACLLAYRNGYTI
TpElo6          AITIALIYIAFVILGSAVMQSLPAMDPYPIKFLYNVSQIFLCAYMTVEAGFLAYRNGYTV
OtElo6          FYLVTVFLWYGRLTRSSDKKIREPTWLRRFIICHNAFLIVLSLYMCLGCVAQAYQNGYTL
                   :::.     :  *   :   .     : : :*.  *.*. ** : .   **:****:

FcElo6          MPCVGYNRDDPAIGNLLWLFYVSKVWDFWDTIFIVLGKKWRQLSFLHVYHHTTIFLFYWL
TpElo6          MPCNHFNVNDPPVANLLWLFYISKVWDFWDTIFIVLGKKWRQLSFLHVYHHTTIFLFYWL
OtElo6          WG-NEFKATETQLALYIYIFYVSKIYEFVDTVIMLLKNNLRQVSFLHIYHHSTISFIWWI
                     ::  :. :.  :::**:**:::* ** :::* :: **:****:***:** :::*:

FcElo6          NANVFYDGDIYLTIALNGFIHTVMYTYYFICMHTKDKKTGKSLPIWWKSSLTLLQLFQFI
TpElo6          NANVLYDGDIFLTILLNGFIHTVMYTYYFICMHTKDSKTGKSLPIWWKSSLTAFQLLQFT
OtElo6          IARRAPGGDAYFSAALNSWVHVCMYTYYLLSTLIGKEDPKRSNYLWWGRHLTQMQMLQFF
                 *.   .** :::  **.::*. *****::.    .... :*  :**   ** :*::**

FcElo6          TMMSQGLYLIIFGCESLSIRVTATYVVYILSLFFLFAQFFVASYMQPKKSKTA-ELGTLI
TpElo6          IMMSQATYLVFHGCDKVSLRITIVYFVSLLSLFFLFAQFFVQSYMAPKKKKSA-------
OtElo6          FNVLQALYCASF--STYPKFLSKILLVYMMSLLGLFGHFYYSKHIAAAKLQKKQQ-----
                  : *. *   .  .. .  ::   .* ::**: **.:*:  .:: . * :.
```

**Figure S4.** Codon-optimised nucleotide sequences. (**A**), Ost 809D6; (**B**), Ost809D4; (**C**) Fc ELO6.

**A**

```
1 ATGCGTGTGGAAACCGAAGACGATAATGTGCCAACTGTTACTGTGGGATTGTCAGAGGAG
 61 TCCGATGGAATGAAGGGAGCAAGGAACCCCGGAGCACGTGCTTGGAAGTCGACGTTGGAG
121 CCGCACGCCGTGGCAAAGTCATTCGATCGTAGGTGGGTTAAGGTTGACGGAGTCGAATAC
181 GACGTAACTGATTTCAAGCATCCCGGAGGATCAGTTATCTACTATATGCTTTCTAACACC
241 GGAGCTGATGCCACTGAGGCTTTCAAGGAATTTCACTATCGTAGTAAGAAGGCCAGGAAG
301 GCACTTGCTGCCCTCCCACAACGTGAGCCTGAAGACGCTTCGCCAGTCGAGGATGCCAAT
361 ATGCTCAAGGACTTCGCAAAGTGGCGTAAGGATTTGGAGAGGGAAGGATTCTTTAAGCCA
421 AGTCCTGCTCACGTGGCCTACCGTTTCGCCGAACTCGCAGCTATGTTTGCTTTGGGAACT
481 GCCCTTATGTATGCACGTTGGCATGCTACGTCTGTCTTCGTAACAGCCTGTTTCTTTGGA
541 GCAAGGTGTGGATGGGTGCAACACGAGGGAGGACATTCTTCCTTGACCGGATCCATCTGG
601 TGGGATAAGCGTATTCAGGCATTCACTGCTGGATTTGGACTTGCCAGTTCGGGAGACATG
661 TGGAACCTCATGCACAATAAGCACCATGCAACGCCACAAAAAGTTAGGCATGATATGGAC
721 CTCGATACCACTCCTGCAGTGGCTTTCTTTAACACAGCTGTTGAGGAAAATCGTCCTAGG
781 AAGTTCTCTAAGTTGTGGCTTCGTGTCCAGGCCTGGACCTTTGTGCCCGTTACTTCCGGA
841 TTGGTACTCTTGGCATGGATGTACCTTCTCCACCCGCGTCATATCGCTCGTAGGAAGAAC
901 TATGAGGAAGCCGCATGGATTGTGGCTGCCCATGTTATCAGGACCTCCGTCATTAAGGCT
961 GTAACGGGATACAGTTGGATCACATGTTATGGACTCTTCTTGTCGACTATGTGGGTCTCA
1021 GGATGCTACCTCTTCGCTCACTTTTCAACGTCTCACACACATTTGGACGTGGTTCCATCT
1081 GATAAGCACCTTTCCTGGGTGCGTTACGCCGTTGATCATACCATCGACATTGATCCTTCC
1141 AAGAGTGTCGTAAACTGGCTCATGGGATATTTGAACTGTCAGGTTATCCACCATTTGTTC
1201 CCCGACATGCCGCAATTTCGTCAGCCCGAAGTCAGTCGTAGGTTCGTATCGTTTGCCAAG
1261 AAGTGGAACCTTAATTACAAGGTCATGTCTTACTATGGAGCCTGGAAGGCAACCTTCGGA
1321 AATCTCAACGAAGTCGGAAAGCACTACTACATCCAAGGAAGTCAAATCACAAAGAAGACG
1381 GTTTAG
```

**Figure S4.** *Cont.*

**B**

```
1 ATGCCAACTACTCGTTCTCGTGCTCGTGTTACTACTCCACCTCGTGAAACTCCTACTCGT

 61 GCTAATACTGTTGCTGCTTTAGATCCAGAACGTAAATATACACGTATTCGAGGTGTTGTA

121 TATGATGTTACTGATTTTGCTAGTCGACATCCAGGTGGTGCACAATTATTATCTTTATGT

181 GTTGGTCGTGATGCTACAATTTTAGTAGAATCACATCATTTACGACCAGAAGTTGTACAA

241 AAATATTTAAAAACATTACCTGTTGTAGAAGGTGCTGCTGGTGCATTTGGTCCAGAAGAA

301 ACTTTTCCAAAACCTTTAGATAGTGATTTATATCGTAAAATTCAAGGTCGTGTTCGAAAA

361 GAAATTGTAGAACCATTAAAAATGACACGTGGTCGAGAACCTCATGGTCGTGGTTGGTGT

421 GTTTTAGATGCTGGTGTTGTATTAGCTTTCTTTGCTTTTGCATTAGGTGTTTATTGGAAA

481 ACACCAACTGTAGCTACTGGTTGTTTATTAGGTTTAGCAGGTTATTGGTCTGGTACAGGT

541 TTACAACATACTGCTAATCATGGTGGTTTAGCAAAATCAGGTTTTTGGAATCAATTTTGG

601 GGTTGGTTAGGAAATGATGTTGCTATTGGTAAATCAAGTGTAGAATGGCGTTATCATCAT

661 ATGGTTTCACATCATAGTTATTGTAATGATGCTGATTTAGATCAAGATGTTTATACAGCA

721 TTACCATTATTACGTTTAGATCCTTCACAAGAATTAAAATGGTTTCATCGTTATCAAGCA

781 TTTTATGCACCTTTAATGTGGCCTATGTTATGGTTAGCTGCACAATTTGGTGATGCTCAA

841 AATATTTTAGTTGATAAAGCAAGTCCAGGTGTAGAATATAAAGGTTTAATGAAATTAGAA

901 GTTGCTTTATATGTATTAGGAAAATTTTTACATTTTTCTTTATTATTAGGTGTTCCTGCA

961 TATTTACATGGTTTTGCTAATGCAATTGTACCATTTATTGCTTATGGTGCATTTGGTTCA

1021 TTTGTTTTATGTTGGTTTTTCATTGTAAGTCATAATTTAGAAGCATTAACACCAATTAAT

1081 TTATCTAAATCAACTAAAAATGATTGGGGTGCTTGGCAAATTGAAACTAGTGCATCTTGG

1141 GGTAATGGTTTTTGGTCATTTTTCTCAGGTGGTTTAAATTTACAAATTGAACATCATTTA

1201 TTTCCTGGTTGTGCTCATAATTTATATCCAAAAATGGTTCCTATTATTAAAGAAGAATGT

1261 GAAAAAGCAGGTGTTACATATACTGGTTATGGTGGTTATTTTGGTTTATTACCAATTACT

1321 CGTGATATGTTTGCTTATTTATATAAAATGGGTCGTCAATCTAAAAAATCTGCTTAA
```

**Figure S4.** *Cont.*

**C**

```
1 ATGGATGAGTACAAGGCCACTTTGGAGTCAGTAGGAGACGCTATTATTCAATGGGCAGAC

61 CCCGAGTCACAGTTCACCGGATTCACTAAGGGATGGTTCCTCACCGATTTTACTTCAGCT

121 TTCTCTATCGCCCTCGTCTACGTATTGTTCGTGATCATTGGATCCCAAGTGATGAAGGTT

181 CTCCCTGCTATCGACCCATACCCTATCAAGTTCTTTTATAACGTTAGTCAGATCATGTTG

241 TGTGCCTATATGACCATTGAGGCATGCCTCTTGGCTTACCGTAATGGATATACTATCATG

301 CCCTGTGTCGGATACAACAGGGATGACCCGGCAATTGGAAATCTTCTCTGGCTTTTCTAT

361 GTCTCGAAAGTATGGGATTTTTGGGACACGATCTTCATTGTGTTGGGAAAGAAGTGGCGT

421 CAATTGTCATTTCTTCACGTTTACCACCATACCACTATCTTCCTCTTCTACTGGCTCAAC

481 GCCAATGTCTTCTACGATGGAGACATCTATTTGACCATTGCACTTAACGGATTTATCCAC

541 ACGGTAATGTACACATACTACTTCATCTGTATGCATACCAAGGATAAGAAGACTGGAAAG

601 AGTCTCCCAATCTGGTGGAAGTCTTCCCTCACGTTGCTTCAATTGTTCCAGTTCATCACA

661 ATGATGTCTCAGGGACTCTACTTGATCATTTTCGGATGCGAAAGTTTGTCGATCAGGGTG

721 ACGGCCACATACGTGGTTTATATTCTTTCTCTCTTCTTTTTGTTCGCCCAGTTTTTCGTC

781 GCCTCCTACATGCAGCCCAAGAAGTCCAAGACAGCCTGA
```

**References**

1. Hoffmann, M.; Wagner, M.; Abbadi, A.; Fulda, M.; Feussner, I. Metabolic engineering of omega3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. *J. Biol. Chem*. 2008, *283*, 22352–22362.

2. Petrie, J.R.; Shrestha, P.; Mansour, M.P.; Nichols, P.D.; Liu, Q.; Singh, S.P. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Delta6-desaturase with omega-3 preference from the marine microalga *Micromonas pusilla*. *Metab. Eng.* 2010, *12*, 233–240.

3. Petrie, J.R.; Liu, Q.; Mackenzie, A.; Shrestha, P.; Mansour, M.P.; Robert, S.S.; Frampton, D.F.; Blackburn, S.I.; Nichols, P.D.; Singh, S.P. Isolation and characterization of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. *Mar. Biotechnol*. 2010, *12*, 430–438.

4. Domergue, F.; Abbadi, A.; Zähringer, U.; Moreau, H.; Heinz, E. *In vivo* characterization of the first acyl-CoA Delta6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. *Biochem. J.* 2005, *389*, 483–490.

5. Meyer, A.; Kirsch, H.; Domergue, F.; Abbadi, A.; Sperling, P.; Bauer, J.; Cirpus, P.; Zank, T.K.; Moreau, H.; Roscoe, T.J.; *et al.* Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res*. 2004, *45*, 1899–1909.

6. Ahman, K.; Heilmann, M.; Feussner, I. Identification of a Δ4-desaturase from microalga *Ostreococcus lucimarinus*. *Eur. J. Lipid Sci. Technol*. 2011, *113*, 832–840.

7. Sayanova, O.; Haslam, R.; Venegas-Calerón, M.; Ruiz-Lopez, N.; Worthy, C.; Rooks, P.; Allen, M.J.; Napier, J.A. Identification and functional characterization of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore *Emiliania huxleyi*. *Phytochemistry* **2011**, *72*, 594–600.

8. Tonon, T.; Sayanova, O.; Michaelson, L.V.; Qing, R.; Harvey, D.; Larson, T.R.; Li, Y.; Napier, J.A.; Graham, I.A. Fatty acid desaturases from the microalga *Thalassiosira pseudonana*. *FEBS J.* **2005**, *272*, 3401–3412.

# List of Abbreviations

| | |
|---|---|
| ACCase | Acetyl-CoA carboxylase |
| ACP | Acyl carrier protein |
| ADHD | Attention-deficit hyperactivity disorder |
| ADP | Adenosine diphosphate |
| AGPase | ADP-glucose pyrophosphorylase |
| ALA | α-Linoleic acid |
| ANOVA | Analysis of variance |
| ARA | Arachidonic acid |
| ATP | Adenosine triphosphate |
| BODIPY | Boron-dipyrromethene |
| bp | Base pairs |
| cDNA | Complementary deoxyribonucleic acid |
| CoA | Coenzyme A |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CVD | Cardiovascular disease |
| DAG | Diacylglycerol |
| DGAT | Diacylglycerol acyltransferase |
| DGLA | Di-homo γ-Linoleic acid |
| DGTS | 1, 2-diacylglyceryl-3-O-4'-(N, N, N-trimethylhomoserine) |
| DHA | Docosahexaenoic acid |
| DHAP | Dihydroxyacetone phosphate |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPA | Docosapentaenoic acid |
| DPA n-6 | Docosapentaenoic acid |
| dsRNA | Double-strand ribonucleic acid |
| DTA | Docosatetraenoic acid |
| ENR | Enoyl-ACP reductase |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| ESAW | Enriched Seawater, Artificial Water |

| | |
|---|---|
| EST | Expressed sequence tag |
| ETA | Eicosatetraenoic acid |
| FA | Fatty acid |
| FAD | Fatty acid desaturase |
| FAT | Fatty acyl-ACP thioesterase |
| FID | Flame ionisation detector |
| G3PDH | Gycerol-3-phosphate dehydrogenase |
| GC | Gac chromatography |
| GC-FID | Gas chromatography - flame ionisation detector |
| GC-MS | Gas chromatography–mass spectrometry |
| GFP | Green fluorescent protein |
| GLA | γ-Linoleic acid |
| GM | Genetically modified |
| GMO | Genetically modified organism |
| GPAT | Glycerol-3-phosphate acyltransferase |
| HD | 3-hydroxyacyl-ACP dehydratase |
| JGI | US DOE Joint Genome Institute |
| KAR | 3-ketoacyl-ACP reductase |
| KAS | 3-ketoacyl-ACP synthase |
| LA | Linoleic acid |
| LACS | Long-chain acyl-CoA synthetases |
| LB | Lipid body |
| LC | Liquid chromatography |
| LCAT | Lecithin:cholesterol acyltransferases |
| LC-MS | Liquid chromatography-mass spectrometry |
| LC-PUFA | Long-chain polyunsaturated fatty acid |
| LPAAT | Lyso-phosphatidic acid acyltransferase |
| LPAT | Lyso-phosphatidylcholine acyltransferase |
| MAT | Malonyl-CoA:ACP transacylase |
| MGDG | Monogalactosyldiacylglycerol |
| MRM | Multi reaction monitoring |
| mRNA | Messenger ribonucleic acid |
| MS | Mass spectrometry |
| MUFA | Monounsaturated fatty acid |
| ORF | Open reading frame |

| | |
|---|---|
| PA | Phosphatidic acid |
| PAP | Phosphatidic acid phosphatase |
| PC | Phosphatidylcholine |
| PCR | Polymerase chain reaction |
| PDAT | Phospholipid:diacylglycerol acyltransferase |
| PDH | Pyruvate dehydrogenase complex |
| PE | Phosphatidylethanolamine |
| PEG | Polyethylene glycol |
| PG | Phosphatidylglycerol |
| PGD | Plastid galactoglycerolipid degradation |
| PI | Phosphatidylinositol |
| PS | Phosphatidylserine |
| PUFA | Polyunsaturated fatty acid |
| RCF | Relative centrifugal force |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| RPM | Rotation per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SCO | Single cell oil |
| SDA | Stearidonic acid |
| SD-Ura | Synthetic dextrose minimal medium minus uracil |
| siRNA | Small interfering ribonucleic acid |
| SQDG | Sulfoquinovosyldiacylglycerol |
| TAG | Triacylglycerol |
| TALE | Transcription activator-like effector |
| TALEN | Transcription activator-like effector nuclease |
| T-DNA | Transfer DNA |
| TFA | Total fatty acids |
| Ti | Tumour inducing |
| VLC-FA | Very long-chain fatty acid |
| VLC-PUFA | Very long-chain polyunsaturated fatty acid |
| WT | Wild type |
| ZFN | Zinc finger nuclease |
| ω3 / ω6 | Omega 3 / Omega 6 |

# References


Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zähringer U, Cirpus P, Heinz E (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: Constraints on their accumulation. *Plant Cell*, 16: 2734–2748

Ahmad I, Sharma AK, Daniell H, Kumar S (2014) Altered lipid composition and enhanced lipid production in Microalgae by introduction of brassica diacylglycerol acyltransferase 2. *Plant Biotechnol*. J., doi: 10.1111/pbi.12278

Ahman K, Heilmann M, Feussner I (2011) Identification of a Δ4-desaturase from microalga *Ostreococcus lucimarinus*. *Eur. J. Lipid Sci. Technol*., 113: 832–840

Aliyu-Paiko M, Hashim R, Shu-Chien AC (2011) Crude palm oil is a sustainable alternative to the growing fish oil scarcity particularly for the aquaculture of warm freshwater fish species. *Adv. Aquac. Around World*, 30

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J. Mol Biol.*, 215(3): 403-410

Amato A (2010) Diatom Reproductive Biology: Living in a Crystal Cage. *Int. J. Plant. Repr. Biol.*, 2(1): 1-10

An M, Mou S, Zhang X, Ye N, Zheng Z, Cao S, Xu D, Fan X, Wang Y, Miao J (2013) Temperature regulates fatty acid desaturases at a transcriptional level and modulates the fatty acid profile in the Antarctic microalga *Chlamydomonas* sp. *ICE-L. Bioresource technology*, 134: 151-157

Apt KE, Kroth-Pancic PG, Grossman AR (1996) Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Mol. Gen. Genet*., 252(5): 572-579

Apt KE, Zaslavkaia L, Lippmeier JC, Lang M, Kilian O, Wetherbee R, Grossman AR, Kroth PG (2002) In vivo characterization of diatom multipartite plastid targeting signals. *J. Cell Sci*. 115(21): 4061-4069

Archibald JM, Rogers MB, Toop M, Ishida K, Keeling PJ (2003) Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga Bigelowiella natans. *Proc. Natl. Acad. Sci. USA*, 100: 7678-7683

Armbrust EV (1999) Identification of a New Gene Family Expressed during the Onset of Sexual Reproduction in the Centric Diatom *Thalassiosira weissflogii*. *Appl. Environ. Microb.*, 65(7): 3121-3128

Armbrust EV, Galindo HM (2001) Rapid evolution of a sexual reproduction gene in centric diatoms of the genus Thalassiosira. *Appl. Environ. Microb.*, 67(8): 3501-3513

Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kroger N, Lau WW, Lane TW, Larimer FW, Lippmeier JC, Lucas S, Medina M, Montsant A, Obornik M, Parker MS, Palenik B, Pazour GJ, Richardson PM, Rynearson TA, Saito MA, Schwartz DC, Thamatrakoln K, Valentin K, Vardi A, Wilkerson FP, Rokhsar DS (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 306: 79–86

Ashworth J, Coesel S, Lee A, Armbrust EV, Orellana MV, Baliga NS (2013). Genome-wide diel growth state transitions in the diatom *Thalassiosira pseudonana*. *P. Natl. Acad. Sci. USA*, 110(18): 7518-7523

Atkinson A, Siegel S, Pakhomov EA, Jessopp MJ, Loeb V (2009) A re-appraisal of the total biomass and annual production of Antarctic krill Deep Sea Research Part I. *Oceanogr. Res. Pap.*, 56 (5): 727–740

Austin JR, Frost E, Vidi PA, Kessler F, Staehelin LA (2006) Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. *Plant Cell*, 18: 1693–1703

Babu MM, Teichmann SA (2003) Evolution of transcription factors and the gene regulatory network in *Escherichia coli*. *Nucleic Acids Res*., 31: 1234–1244

Bachvaroff TR, Concepcion GT, Rogers CR, Herman EM, Delwiche CF (2004) Dinoflagellate expressed sequence tag data indicate massive transfer of chloroplast genes to the nuclear genome. *Protist*., 155: 65-78

Ball SG, Morell MK (2003) From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annu. Rev. Plant Biol*. 54: 207–233

Barclay WR, Meager KM, Abril JR (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J. App. Phycol.*, 6(2): 123-129

Bates PD, Johnson SR, Cao X, Li J, Nam JW, Jaworski JG, Ohlrogge JB, Browse J (2014) Fatty acid synthesis is inhibited by inefficient utilization of unusual fatty acids for glycerolipid assembly. *Proc. Natl. Acad. Sci. USA*, 111: 1204–1209

Baulcombe D (2004) RNA silencing in plants. *Nature*, 431: 356–363

Beisson F, Li Y, Bonaventure G, Pollard M, Ohlrogge JB (2007) The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of Arabidopsis. *Plant Cell*, 19: 351–368

Benning C, Ohta H (2005) Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. *J. Biol. Chem*., 280: 2397–2400

Benning C (2008) A role for lipid trafficking in chloroplast biogenesis. *Prog. Lipid Res*., 47: 381–389

Benning C (2009) Mechanisms of lipid transport involved in organelle biogenesis in plant cells. In: Annual Review of Cell and Developmental Biology, Vol. 25, Annual Reviews, Palo Alto, CA: 71–91

Bergen WG, Mersmann HJ (2005) Comparative aspects of lipid metabolism: impact on contemporary research and use of animal models. *J. Nurt.*, 135(11): 2499-2502

Berges JA, Franklin DJ, Harrison PJ (2001) Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the past two decades. *J. Phycol*., 37: 1138–1145

Berthold P, Schmitt R, Mages W (2002) An engineered Streptomyces hygroscopicus aph 7" gene mediates dominant resistance against hygromycin B in *Chlamydomonas reinhardtii*. *Protist*, 153(4): 401–412

Beurdeley M, Bietz F, Li J, Thomas S, Stoddard T, Juillerat A, Zhang F, Voytas DF, Duchateau P, Silva GH (2013) Compact designer TALENs for efficient genome engineering. *Nature Comm.*, 4: 1762

Bevan MW, Flavell RB & Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature*, 304: 184-187

Bimboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*., 7: 1513 – 1523

Blowers AD, Bogorad L, Shark KB, Sanford JC (1989) Studies on Chlamydomonas chloroplast transformation: foreign DNA can be stably maintained in the chromosome. *Plant Cell*., 1: 123-132

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. *Science*, 326(5959): 1509-1512

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. *J. Mol. Biol*., 312(3): 425–438

Bouget FY, Lefranc M, Thommen Q, Pfeuty B, Lozano JC, Schatt P, Botebol H, Vergé V (2014) Transcriptional versus non-transcriptional clocks: a case study in Ostreococcus. *Mar. Genomics*, 14: 17–22

Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K, Kuo A, Maheswari U, Martens C, Maumus F, Otillar RP, Rayko E, Salamov A, Vandepoele K, Beszteri B, Gruber A, Heijde M, Katinka M, Mock T, Valentin K, Verret F, Berges JA, Brownlee C, Cadoret JP, Chiovitti A, Choi CJ, Coesel S, De Martino A, Detter JC, Durkin C, Falciatore A, Fournet J, Haruta M, Huysman MJ, Jenkins BD, Jiroutova K, Jorgensen RE, Joubert Y, Kaplan A, Kröger N, Kroth PG, La Roche J, Lindquist E, Lommer M, Martin-Jézéquel V, Lopez PJ, Lucas S, Mangogna M, McGinnis K, Medlin LK, Montsant A, Oudot-Le Secq MP, Napoli C, Obornik M, Parker MS, Petit JL, Porcel BM, Poulsen N, Robison M, Rychlewski L, Rynearson TA, Schmutz J, Shapiro H, Siaut M, Stanley M, Sussman MR, Taylor AR, Vardi A, von Dassow P, Vyverman W, Willis A, Wyrwicz LS, Rokhsar DS, Weissenbach J, Armbrust EV, Green BR, Van de Peer Y, Grigoriev IV (2008) The Phaeodactylum genome reveals the evolutionary history of diatom genomes. *Nature*, 456: 239-244

Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ, Gallaher SD, Johnson S, Benning C, Pellegrini M, Grossman A, Merchant SS (2012) Three acyltransferases and nitrogen-responsive regulator are implicated in

nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. *J. Biol. Chem*. 287: 15811–15825

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark KB, Sanford JC (1988) Chloroplast transformation in Chlamydomonas with high velocity microprojectiles. *Science* 240: 1534-1537

Bozhkov AI, Menzyanova NG, Kovaleva MK, Pyatak NI (2014) Growth of Dunaliella in "ideal" conditions retains annual variability of biochemical features. *Universal Journal of Plant Science*, 2(2): 31-39

Braun R, Farré EM, Schurr U, Matsubara S (2014), Effects of light and circadian clock on growth and chlorophyll accumulation of *Nannochloropsis gaditana*. *J. Phycol.*, 50: 515–525

Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. *Trends Genet*., 22: 268–280

Broun P, Gettner S, Somerville C (1999) Genetic engineering of plant lipids. *Annu. Rev. Nutr*., 19: 197–216

Bruening G Lyons JM (2000) The case of the FLAVR SAVR tomato. *Cal. Agric.*, 54(4):6-7

Calder PC (2003) N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids*, 38: 343–352

Carroll D (2011) Genome engineering with zinc-finger nucleases. *Genetics*, 188(4): 773-782

Carroll SB (2000) Endless forms: the evolution of gene regulation and morphological diversity. *Cell*, 101: 577–580

Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell*, 136: 642–655

Casas-Mollano JA, Rohr J, Kim EJ, Balassa E, van Dijk K, Cerutti H (2008) Diversification of the core RNA interference machinery in *Chlamydomonas reinhardtii* and the role of DCL1 in transposon silencing. *Genetics*, 179(1): 69-81

Cerutti H, Johnson AM, Gillham NW, Boynton JE (1997) A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. *Genetics*, 145(1): 97-11

Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. *Nat. Rev. Genet*., 8: 884–896

Chapman KD, Ohlrogge JB (2012) Compartmentation of triacylglycerol accumulation in plants. *J. Biol. Chem*., 287: 2288–2294

Chen GQ (2007) Lipid and fatty acid composition and their biosyntheses in relation to carotenoid accumulation in the microalgae *Nitzschia laevis* (Bacillariophyceae) and *Haematococcus pluvialis* (chlorophyceae). In: PhD Thesis, The University of Hong Kong, Hong Kong

Chen HL, Li SS, Huang R, Tsai HJ (2008) Conditional production of a functional fish growth hormone in the transgenic line of *Nannochloropsis oculata* (Eustigmatophyceae). *J. Phycol*., 44: 768–776

Chen Y, Wang Y, Sun Y, Zhang L, Li W (2001) Highly efficient expression of rabbit neutrophil peptide-1 gene in *Chlorella ellipsoidea* cells. *Curr. Genet*., 39(5-6): 365-370

Chen YQ, Kuo MS, Li S, Bui HH, Peake DA, Sanders PE, Thibodeaux SJ, Chu S, Qian YW, Zhao Y, Bredt DS, Moller DE, Konrad RJ, Beigneux AP, Young SG, Cao G (2008) AGPAT6 is a novel microsomal glycerol-3-phosphate acyltransferase. *J. Biol. Chem*. 283: 10048–10057

Cheney D, Metz B, Stiller J (2001) Agrobacterium-mediated genetic transformation in the macroscopic marine red alga *Porphyra yezoensis. J. Phycol*. 37(Suppl.): 11

Chepurnov VA, Mann DG, Sabbe K, Vyverman W (2004) Experimental studies on sexual reproduction in diatoms. *Int. Rev. Cytol*, 237: 91-154

Chilton MD, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell*, 11: 263-271

Choquet Y, Stern DB, Wostrikoff K, Kuras R, Girard-Bascou J, Wollman FA (1998) Translation of cytochrome f is autoregulated through the 5' untranslated region of petA mRNA in *Chlamydomonas chloroplasts*. *Proc. Natl. Acad. Sci. USA*, 95: 4380–4385

Chow KC, Tung WL (1999) Electrotransformation of *Chlorella vulgaris. Plant Cell Rep*., 18: 778–780

Chungjatupornchai W, Watcharawipas A (2014) Diacylglycerol acyltransferase type 2 cDNA from the oleaginous microalga *Neochloris oleoabundans*: cloning and functional characterization. *J. Appl. Phycol.*, doi: 10.1007/s10811-014-0448-6

Coleman RA, Lee DP (2004) Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid Res*., 43: 134–176

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, 339(6121): 819-823

Constable AJ (2011) Lessons from CCAMLR on the implementation of the ecosystem approach to managing fisheries. *Fish Fish*, 12: 138–151

Crépineau F, Roscoe T, Kaas R, Kloareg B, Boyen C (2000) Characterisation of complementary DNAs from the expressed sequence tag analysis of life cycle stages of *Laminaria digitata* (Phaeophyceae). *Plant Mol. Biol.*, 43: 503-513

Cronan Jr JE, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. *Prog. Lipid Res*., 41: 407–435

Cushman SW (1970) Structure-function relationships in adipose cell. 1. Ultrastructure of isolated adipose cell. *J. Cell Biol*., 46: 326–341

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne H (2000) Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc. Natl. Acad. Sci. USA*, 97: 6487–6492

Dawson HN, Burlingame R, Cannons AC (1997) Stable Transformation of Chlorella: Rescue of Nitrate Reductase-Deficient Mutants with the Nitrate Reductase Gene. *Curr. Microbiol*., 35(6): 356-362

De Riso V, Raniello R, Maumus F, Rogato A, Bowler C, Falciatore A (2009) Gene silencing in the marine diatom *Phaeodactylum tricornutum. Nucleic Acids Res.*, 37(14): e96. doi: 10.1093/nar/gkp448

Demir-Hilton E, Sudek S, Cuvelier ML, Gentemann CL, Zehr JP, Worden Z (2011) Global distribution patterns of distinct clades of the photosynthetic picoeukaryote Ostreococcus. *ISME J*., 5: 1095–1107

Deng XD, Gu B, Li YJ, Hu XW, Guo JC, Fei XW (2012) The roles of acyl-CoA: diacylglycerol acyltransferase 2 genes in the biosynthesis of triacylglycerols by the green algae *Chlamydomonas reinhardtii*. *Mol. Plant*, 5: 945–947

Depauw FA, Rogato A, Ribera d'Alcalá M, Falciatore A (2012) Exploring the molecular basis of responses to light in marine diatoms*. J. Exp. Bot*., 63: 1575–1591

Depicker A, Montagu MV, Schell J (1978) Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity. *Nature*, 275: 150-153

Derelle E, Ferraz C, Rombauts S, Rouzé P, Worden AZ, Robbens S, Partensky F, Degroeve S, Echeynié S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C, Panaud O, Piégu B, Ball SG, Ral JP, Bouget FY, Piganeau G, De Baets B, Picard A, Delseny M, Demaille J, Van de Peer Y, Moreau H (2006) Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc. Natl. Acad. Sci. USA*, 103: 11647-11652

Ding J, Li X, Hu H (2012) Systematic prediction of cis-regulatory elements in the *Chlamydomonas reinhardtii* genome using comparative genomics. *Plant Physiol*., 160: 613–623

Doetsch NA, Favreau MR, Kuscuoglu N, Thompson MD, Hallick RB (2001) Chloroplast transformation in *Euglena gracilis*: splicing of a group III twintron transcribed from a transgenic psbK operon. *Curr. Genet.*, 39(1): 49-60

Domergue F, Lerchl J, Zahringer U, Heinz E (2002) Cloning and functional characterization of *Phaeodactylum tricornutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. *Eur. J. Biochem*., 269: 4105–4113

Domergue F, Spiekermann P, Lerchl J, Beckmann C, Kilian O, Kroth PG, Boland W, Zähringer U, Heinz E (2003) New insight into *Phaeodactylum tricornutum* fatty acid metabolism: cloning and functional characterization of plastidial and microsomal Δ12-fatty acid desaturases. *Plant Physiol*., 131: 1648–1660

Domergue F, Abbadi A, Zähringer U, Moreau H, Heinz E (2005) In vivo characterization of the first acyl-CoA Δ6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri. Biochem. J.*, 389: 483–490

Dunahay TG (1993) Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. *Biotechniques*, 15: 452–455, 457–458, 460

Dunahay TG, Jarvis EE, Roessler PG (1995) Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila. J. Phycol*., 31: 1004–1011

Dunahay TG, Jarvis EE, Dais SS, Roessler PG (1996) Manipulation of microalgal lipid production using genetic engineering. *Appl. Biochem. Biotechnol.*, 57: 223–231

Dunwell JM (2014), Genetically modified (GM) crops: European and transatlantic divisions. *Mol. Plant Path.*, 15: 119–121

Durrett TP, McClosky DD, Tumaney AW, Elzinga DA, Ohlrogge J, Pollard M (2010) A distinct DGAT with sn-3 acetyltransferase activity that synthesizes unusual, reduced-viscosity oils in Euonymus and transgenic seeds. *Proc. Natl. Acad. Sci. USA*, 107: 9464–9469

Eastmond PJ, Quettier AL, Kroon JTM, Craddock C, Adams N, Slabas AR (2010) Phosphatidic Acid Phosphohydrolase 1 and 2 regulate phospholipid synthesis at the endoplasmic reticulum in Arabidopsis. *Plant Cell*, 22: 2796–2811

Eichler-Stahlberg A, Weisheit W, Ruecker O, Heitzer M (2009) Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. *Planta*, 229(4): 873-883

Eisenstein M (2013) Plant breeding: Discovery in a dry spell. *Nature*, 501: S7–S9

Elble R (1992) A simple and efficient procedure for transformation of yeasts. *Biotechniques*, 13: 18–20

El-Sheekh MM (1999) Stable transformation of the intact cells of *Chlorella kessleri* with high velocity microprojectiles. *Biol. Plant*., 42: 209–216

Ender F, Godl K, Wenzl S, Sumper M (2002) Evidence for autocatalytic cross-linking of hydroxyproline-rich glycoproteins during extracellular matrix assembly in Volvox. *Plant Cell*, 14(5): 1147-1160

Fábregas J, Maseda A, Domínguez A, Ferreira M, Otero A (2002) Changes in the cell composition of the marine microalga, *Nannochloropsis gaditana*, during a light:dark cycle. *Biotechnol. Lett*., 24: 1699–1703

Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C (1999) Transformation of nonselectable reporter genes in marine diatoms. *Marine Biotech*., 1: 239-251

Fan JL, Andre C, Xu CC (2011) A chloroplast pathway for the de novo biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii. FEBS Lett.*, 585: 1985–1991

Feng S, Xue L, Liu H, Lu P (2009) Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Mol. Biol. Rep*., 36: 1433–1439

Fischer H, Robl I, Sumper M, Kröger N (1999) Targeting and covalent modification of cell wall and membrane proteins heterologously expressed in the diatom *Cylindrotheca fusiformis* (Bacillariophyceae). *J. Phycol.*, 35: 113–120

Food And Agriculture Organization Of The United Nations (FAO) (1986) The Production of Fishmeal and Oil. FAO Fisheries Technical Paper 142 rev. 1. *Food and Agriculture Organization of the UN Rome Italy*, rev 1, 63 pages

Food And Agriculture Organization Of The United Nations (FAO) (2014) The State of World Fisheries and Aquaculture Report: Opportunities and Challenges, Rome. 243 pages

Fourrier N, Bédard J, Lopez-Juez E, Barbrook A, Bowyer J, Jarvis P, Warren G, Thorlby G (2008), A role for SENSITIVE TO FREEZING2 in protecting chloroplasts against freeze-induced damage in Arabidopsis. *The Plant Journal*, 55: 734–745

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA*, 80: 4803-4807

Franklin S, Ngo B, Efuet E, Mayfield SP (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *Plant J*., 30(6): 733-744

Fresco LO (2013) The GMO stalemate in Europe. *Science*, 339, 883

Frewer LJ, van der Lans IA, Fischer ARH, Reinders MJ, Menozzi D, Zhang X, van den Berg I, Zimmermann KL (2013) Public perceptions of agri-food applications of genetic modification – a systematic review and meta-analysis. *Trends Food Sci. Technol.*, 30: 142–152

Frost and Sullivan (2014) Market Report: Global Omega-3 and Omega-6 Polyunsaturated Fatty Acid Ingredients Market: Focus on the Impact of Microalgae Feedstock on the Supply and Demand for Omega-3 and Omega-6 Ingredients. July 2014. NE34-88

Fuhrmann M, Oertel W, Hegemann P (1999) A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant J.*, 19(3): 353-361

Fuhrmann M (2002) Expanding the molecular toolkit for *Chlamydomonas reinhardtii* from history to new frontiers. *Protist*, 153(4): 357–364

Fuhrmann M, Hausherr A, Ferbitz L, Schödl T, Heitzer M, Hegemann P (2004) Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. *Plant Mol Biol*., 55(6): 869-881

Galas DJ, Schmitz A (1978) DNAase footprinting a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res*. 5: 3157–3170

Gan SY, Qin S, Othman RY, Yu D, Phang SM (2003) Transient expression of lacZ in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). *J. Appl. Phycol*. 15: 345–349

Gao H, Wright DA, Li T, Wang Y, Horken K, Weeks DP, Yang B, Spalding MH (2014). TALE activation of endogenous genes in *Chlamydomonas reinhardtii*. *Algal Res.*, 5: 52-60

Garces M, Mancha R (1993) One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. *Anal. Biochem*., 211: 139–143

García-Maroto F, Mañas-Fernández A, Garrido-Cárdenas JA, Alonso DL (2006) Substrate specificity of acyl-Δ6-desaturases from Continental versus *Macaronesian Echium* species. *Phytochemistry*, 67: 540–544

Garner MM, Revzin A (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic Acids Res*., 9: 3047–3060

Gbogouri GA, Linder M, Fanni J and Parmentier M (2006) Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes. *Eur. J. Lipid Sci. Technol.*, 108: 766–775

Ghosh AK, Chauhan N, Rajakumari S, Daum G, Rajasekharan R (2009) At4g24160, a Soluble acylcoenzyme A-dependent lysophosphatidic acid acyltransferase. *Plant Physiol*., 151: 869–881

Gigliotti JC, Davenport MP, Beamer SK, Tou JC, Jaczynski J (2011) Extraction and characterisation of lipids from Antarctic krill *Euphausia superb*. *Food Chem*., 125: 1028–1036

Golden SS, Canales SR (2003) Cyanobacterial circadian clocks - Timing is everything. *Nat. Rev. Microbiol*., 1: 191–199

Gong Y, Zhang J, Guo X, Wan X, Liang Z, Hu CJ, Jiang M (2013) Identification and characterization of PtDGAT2B, an acyltransferase of the DGAT2 acyl-coenzyme A: diacylglycerol acyltransferase family in the diatom *Phaeodactylum tricornutum*. FEBS Lett, 587: 481–487

Goodson C, Roth R, Wang ZT, Goodenough U (2011) Structural correlates of cytoplasmic and chloroplast lipid body synthesis in *Chlamydomonas reinhardtii* and stimulation of lipid body production with acetate boost. *Eukaryot. Cell*, 10: 1592–1606

Goodwin TW (1974) Carotenoids and biliproteins. *Algal Phys. Biochem.*, 10: 176-205

Goold H, Beisson F, Peltier G, Li-Beisson Y (2014). Microalgal lipid droplets: composition, diversity, biogenesis and functions. *Plant cell reports*, 1-11

Goto K, Johnson CH (1995) Is the cell division cycle gated by a circadian clock? The case of *Chlamydomonas reinhardtii. J. Cell Biol*., 129: 1061–1069

Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ (2010) Placing microalgae on the biofuels priority list: a review of the technological challenges. *J. R. Soc. Interface*, 7: 703–726

Gruber A, Vugrinec S, Hempel F, Gould SB, Maier UG, Kroth PG (2007) Protein targeting into complex diatom plastids: functional characterisation of a specific targeting motif. *Plant Mol Biol*., 64(5): 519-530

Guiheneuf F, Leu S, Zarka A, Khozin-Goldberg I, Khalilov I, Boussiba S (2011) Cloning and molecular characterization of a novel acyl-CoA:diacylglycerol acyltransferase 1-like gene (PtDGAT1) from the diatom *Phaeodactylum tricornutum*. FEBS J, 278: 3651–3666

Gustafsson C, Govindarajan S, Minshull J (2004) Codon bias and heterologous protein expression. *Trends Biotechnol.*, 22: 346–353

Hackett JD, Scheetz TE, Yoon HS, Soares MB, Bonaldo MF, Casavant TL, Bhattacharya D (2005) Insights into a dinoflagellate genome through expressed sequence tag analysis. *BMC Genomics*, 6:80

Hagen C, Braune W, Bjorn LO (1994) Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Volvocales). III. Action as a sunshade. *J. Phycol*., 30: 241–248

Hamilton ML, Haslam RP, Napier JA, Sayanova O (2014) Metabolic engineering of *Phaeodactylum tricornutum* for the enhanced accumulation of omega-3 long chain polyunsaturated fatty acids. *Metab. Eng.*, 22: 3–9

Hammond LE, Gallagher PA, Wang SL, Hiller S, Kluckman KD, Posey-Marcos EL, Maeda N, Coleman RA (2002) Mitochondrial glycerol-3-phosphate acyltransferase-deficient mice have reduced weight and liver triacylglycerol content and altered glycerolipid fatty acid composition. Mol. Cell. Biol. 22: 8204–8214

Harding LWJ, Heinbokel JF (1984) Periodicities of photosynthesis and cell division: behavior of phase-lagged replicate cultures of *Ditylum brightwellii* in a diurnally varying photic regime. *Mar. Ecol. Prog. Ser.,* 15: 225–32

Harrington GW, Holz Jr GG (1968) The monoenoic and docosahexaenoic fatty acids of a heterotrophic dinoflagellate. *Biochim. Biophys. Acta - Lipids and Lipid Metabolism*, 164 (1): 137–139

Harrison PJ, Waters RE, Taylor FJR (1980) A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. *J. Phycol*., 16: 28–35

Harwood JL (1988) Fatty acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol*., 39: 101–138

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta, Lipids Lipid Metab.*, 1301: 7–56

Hashimoto K, Yoshizawa AC, Okuda S, Kuma K, Goto S, Kanehisa M (2008) The repertoire of desaturases and elongases reveals fatty acid variations in 56 eukaryotic genomics. *J Lipid Res*, 49: 183–191

Haslam RP, Ruiz-Lopez N, Eastmond P, Moloney M, Sayanova O, Napier JA (2013) The modification of plant oil composition via metabolic engineering – better nutrition by design. *Plant Biotechnol. J.*, 11: 157–168

Hasle GR, Heimdal BR (1970) Some species of the centric diatom genus Thalassiosira studied in the light and electron microscopes. In: Gerloff J, Cholnoky JB Diatomaceae II. Beihefte zur Nova Hedwigia 31: 559-589

Hasle GR, Syvertsen EE, Steidinger KA, Tangen K, Thomas CR (1996) Identifying marine diatoms and dinoflagellates. *Academic Press*

Hawkins RL, Nakamura M (1999) Expression of human growth hormone by the eukaryotic alga, Chlorella. *Curr. Microbiol*. 38: 335–341

Haynes CA, Allegood JC, Sims K, Wang EW, Sullards MC, Merril AH (2008) Quantitation of fatty acyl-coenzyme as in mammalian cells by liquid chromatography-electrospray ionisation tandem mass spectrometry. *J. Lipid Res*., 49: 1113–1125

Heitzer M, Eckert A, Fuhrmann M, Griesbeck C (2007) Influence of codon bias on the expression of foreign genes in microalgae. In: R. León, A. Gaván, E. Fernández (Eds.), Transgenic Microalgae as Green Cell Factories. LLC, Landes Bioscience/Springer Science + Business Media, 46–53

Hellmich RL, Hellmich KA (2012) Use and Impact of Bt Maize. *Nat. Ed. Know.*, 3(10):4

Henry IM, Wilkinson MD, Hernandez JM, Schwarz-Sommer Z, Grotewold E, Mandoli DF (2004) Comparison of ESTs from juvenile and adult phases of the giant unicellular green alga *Acetabularia acetabulum*. *BMC Plant Biol*., 4:3

Herrera-Estrella L, de Block M, Messens E, Hernalsteens JP, van Montagu M, Schell J (1983) Chimeric genes as dominant selectable markers in plant cells. *EMBO J.*, 2 (6): 987-995

Hildebrand M, Kim S, Shi D, Scott K, Subramaniam S (2009) 3D imaging of diatoms with ion-abrasion scanning electron microscopy. *J. Struct. Biol.*, 166(3): 316-328

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983). A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature,* 303: 179-180

Hoffmann M, Wagner M, Abbadi A, Fulda M, Feussner I (2008) Metabolic engineering of omega3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. *J. Biol. Chem*., 283: 22352–22362

Holthuis JCM, Levine TP (2005) Lipid traffic: Floppy drives and a superhighway. *Nat. Rev. Mol. Cell Biol.* 6: 209–220

Hong H, Datla N, Reed DW, Covello PS, MacKenzie SL, Qiu X (2002) High-level production of gamma-linolenic acid in *Brassica juncea* using a Δ6 desaturase from Pythium irregular. *Plant Physiol.*, 129: 354–362

Hu J, Wang D, Li WJ, Jing G, Ning K, Xu J (2014) Genome-wide identification of transcription factors and transcription-factor binding sites in oleaginous microalgae Nannochloropsis. *Nat. Sc. Rep*., 4: 5454

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.*, 54: 621–639

Huang X, Weber JC, Hinson TK, Mathieson AC, Minocha SC (1996) Transient expression of the GUS reporter gene in the protoplasts and partially digested cells of *Ulva lactuca* L.(Chlorophyta). *Bot. Mar*., 39: 467–474

Hung CH, Ho MY, Kanehara K, Nakamura Y (2013) Functional study of diacylglycerol acyltransferase type 2 family in *Chlamydomonas reinhardtii*. FEBS Lett, 587: 2364–2370

Hutvagner G, Simard MJ (2008) Argonaute proteins: key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.*, 9: 22–32

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JRJ, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.,* 31(3): 227-229

Inaba T, Schnell DJ (2008) Protein trafficking to plastids: one theme, many variations. *Biochem J*., 413: 15–28

Innis SM (2008) Dietary omega 3 fatty acids and the developing brain. *Brain Res*., 1237: 35–43

Iskandarov U, Khozin-Goldberg I, Ofir R, Cohen Z (2009) Cloning and characterization of the Δ6 polyunsaturated fatty acid elongase from the green microalga *Parietochloris incisa*. *Lipids*, 44: 545–554

Iskandarov U, Khozin-Goldberg I, Cohen Z (2010) Cloning and characterization of the Δ12, Δ6 and Δ5 desaturases from the green microalga *Parietochloris incisa*. *Lipids*, 45: 519–530

Jacobshagen S, Johnson CH (1994) Circadian rhythms of gene expression in *Chlamydomonas reinhardtii*: circadian cycling of mRNA abundances of cab II, and possibly of β-tubulin and cytochrome c. *Eur. J. Cell Biol*. 64: 142–152

Jako C, Kumar A, Wei YD, Zou JT, Barton DL, Giblin EM, Covello PS, Taylor DC (2001) Seedspecific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. *Plant Physiol*., 126: 861–874

Jakobiak T, Mages W, Scharf B, Babinger P, Stark K, Schmitt R (2004) The bacterial paromomycin resistance gene, aphH, as a dominant selectable marker in *Volvox carteri. Protist*, 155(4): 381-393

James C (2012) Global status of commercialized biotech/GM crops. ISAAA Brief No 44

James CM, Al-Hinty S, Salman AE (1989) Growth and omega-3 fatty acid and amino acid composition of microalgae under different temperature regimes. *Aquaculture*, 77: 337–352

James CN, Horn PJ, Case CR, Gidda SK, Zhang DY, Mullen RT, Dyer JM, Anderson RGW, Chapman KD (2010) Disruption of the Arabidopsis CGI-58 homologue produces Chanarin–Dorfman-like lipid droplet accumulation in plants. *Proc. Natl. Acad. Sci. USA*, 107: 17833–17838

Jarvis EE, Brown LM (1991) Transient expression of firefly luciferase in protoplasts of the green alga *Chlorella ellipsoidea. Curr. Genet*., 19: 317–321

Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytol*., 179(2): 257–285

Jaworski J, Cahoon EB (2003) Industrial oils from transgenic plants. *Curr. Opin. Plant. Biol*., 6: 178–84

Jaworski JG, Clough RC, Barnum SR (1989) A cerulenin insensitive short chain 3-ketoacyl acyl carrier protein synthase in *Spinacia oleracea* leaves. *Plant Physiol*., 90: 41–44

Jiang P, Qin S, Tseng CK (2003) Expression of the lacZ reporter gene in sporophytes of the seaweed *Laminaria japonica* (Phaeophyceae) by gametophyte-targeted transformation. *Plant Cell Rep*., 21(12): 1211-1216

Johnson CH, Kondo T, Hastings JW (1991) Action spectrum for resetting the circadian phototaxis rhythm in the CW15 strain of Chlamydomonas. II. Illuminated cells. *Plant Physiol*., 97: 1122–1129

Joint Genome Institute *Fragilariopsis cylindrus* genome project. Available online: http://genome.jgi-psf.org/Fracy1/Fracy1.home.html (accessed on 10 May 2012).

Joint Genome Institute *Ostreococcus* sp. RCC809 2.0 genome project. Available online: http://genome.jgi-psf.org/OstRCC809_2/OstRCC809_2.home.html (accessed on 5 April 2012).

Jones A, Davies HM, Voelker TA (1995) Palmitoylacyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. *Plant Cell*, 7: 359–371

Jothi R, Cuddapah S, Barski A, Cui K, Zhao K (2008) Genome-wide identification of in vivo protein–DNA binding sites from ChIP-Seq data. *Nucleic Acids Res*., 36: 5221–5231

Joung JK, Sander JD (2012). TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Bio.,* 14(1): 49-55

Kalaitzandonakes N, Alston JM, Bradford KJ (2007) Compliance costs for regulatory approval of new biotech crops. *Nat. Biotechnol.*, 25: 509–511

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Kramer R, Linke B, McHardy AC, Meyer F, Mockel B, Pfefferle W, Puhler A, Rey DA, Ruckert C, Rupp O, Sahm H, Wendisch VF, Wiegrabe I, Tauch A (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol*., 104: 5–25

Kalscheuer R, Steinbuchel A (2003) A novel bifunctional wax ester synthase/acyl-CoA: diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *J. Biol. Chem*., 278: 8075–8082

Kalscheuer R, Stolting T, Steinbuchel A (2006) Microdiesel: *Escherichia coli* engineered for fuel production. *Microbiology*, 152: 2529–2536

Kang F, Rawsthorne S (1994) Starch and fatty-acid synthesis in plastids from developing embryos of oilseed rape (Brassica-Napus L). *Plant J*., 6: 795–805

Kellis M, Patterson N, Endrizzi M, Birren B, Lander ES (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature*, 423: 241–254

Kilian O, Benemann CS, Niyogi KK, Vick B (2011) High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proc. Natl. Acad. Sci. USA*, 108: 21265–21269

Kilian O, Vick B (2011) Homologous recombination in an algal nuclear genome. Patent number: US 2011/0091977 A1

Kim HU, Li Y, Huang AH (2005). Ubiquitous and endoplasmic reticulum–located lysophosphatidyl acyltransferase, LPAT2, is essential for female but not male gametophyte development in Arabidopsis. *Plant Cell*, 17(4), 1073-1089

Kindle KL (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. USA*, 87: 1228–1232

Kinney AJ, Cahoon EB, Damude HG, Hitz WD, Kolar CW, Liu ZB (2004) Production of very long chain polyunsaturated fatty acids in oilseed plants. WO 2004/071467 A2

Kinney AJ, Clemente TE (2005) Modifying soybean oil for enhanced performance in biodiesel blends. *Fuel Process. Technol.*, 86(10): 1137-1147

Klinger D, Naylor R (2012) Searching for solutions in aquaculture: charting a sustainable course. *Annu. Rev. Env. Resour.*, 37: 247–276

Kröger N, Poulsen N (2008) Diatoms – from cell wall biogenesis to nanotechnology. *Annu. Rev. Genet*., 42: 83 – 107

Kohlwein SD (2010) Triacylglycerol homeostasis: insights from yeast. *J. Biol. Chem*., 285: 15663–15667

Koo AJK, Ohlrogge JB, Pollard M (2004) On the export of fatty acids from the chloroplast. *J. Biol. Chem*., 279: 16101–16110

Kooistra WHCF, Gersonde R, Medlin LK, Mann DG (2007) The origin and evolution of the diatoms: their adaptation to a planktonic existence. In: Evolution of Primary Producers in the Sea. Falkowski PG, Knoll AH (Ed.) 207–249

Kooistra WHCF, Medlin LK (1996) Evolution of the diatoms (Bacillariophyta): IV. A reconstruction of their age from small subunit rRNA coding regions and the fossil record. *Molec. Phylogenet. Evol.* 6: 391–407

Kubler JE, Minocha SC, Mathieson AC (1994) Transient expression of the GUS reporter gene in protoplasts of *Porphyra miniata* (Rhodophyta). *J. Mar. Biotechnol.*, 1: 165–169

Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV (2004) Genetic transformation of the green alga—*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens. Plant Sci*. 166: 731–738

Kurtzman AM, Cheney DP (1991) Direct gene transfer and transient expression in a marine red alga using the biolistic method. *J. Phycol*., 27(Suppl.): 42

Kyle DJ (1992) Production and use of lipids from microalgae. *Lipid Technol*., 4: 59–64

Kyle DJ (1996) Production and use of a single cell oil which is highly enriched in docosahexanoic acid. *Lipid Technol*., 2**:** 107–110

Kyle DJ (2001) The Large Scale Production and Use of a Single Cell Oil Highly Enriched in DHA. In: Shahidi F & Finley JF (Ed.) *Omega-3 Fatty Acids: Chemistry, Nutrition and Health Effects*. Washington. ACS Symposium Series, 788: 92–107

La Russa M, Bogen C, Uhmeyer A, Doebbe A, Filippone E, Kruse O, Mussgnug JH (2012) Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii. J. Biotechnol*., 162: 13–20

Lapidot M, Raveh D, Sivan A, Arad SM, Shapira M (2002) Stable chloroplast transformation of the unicellular red alga Porphyridium species. *Plant Physiol.*, 129: 7–12

Larson TR, Graham IA (2001) A novel technique for the sensitive quantification of acyl CoA esters from plant tissues. *Plant J*. 25: 115–125

Laurent P, Huang AHC (1992) Organ-specific and development-specific acyl coenzyme-a lysophosphatidate acyltransferases in palm and meadowfoam. *Plant Physiol*. 99: 1711–1715

Li J, Han D, Wang D, Ning K, Jia J, Wei L, Jing X, Huanga S, Chen J, Li Y, Hu Q, Xu J (2014) Choreography of Transcriptomes and Lipidomes of Nannochloropsis Reveals the Mechanisms of Oil Synthesis in Microalgae. *Plant Cell*, 26(4): 1645-1665

Li XB, Moellering ER, Liu BS, Johnny C, Fedewa M, Sears BB, Kuo MH, Benning C (2012) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in *Chlamydomonas reinhardtii*. *Plant Cell*, 24: 4670–4686

Li Y, Beisson F, Koo AJ, Molina I, Pollard M, Ohlrogge J (2007) Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proc. Natl. Acad. Sci. USA*, 104: 18339–18344

Li Y, Han D, Hu G, Dauvillee D, Sommerfeld M, Ball S, Hu Q (2010) Chlamydomonas starchless mutant defective in ADP-glucose pyrophosphorylase hyperaccumulates triacylglycerol. *Metab. Eng*., 12: 387–391

Li Y, Han D, Yoon K, Zhu S, Sommerfeld M, Hu Q (2013) Molecular and cellular mechanisms for lipid synthesis and accumulation in microalgae: biotechnological implications. In: Richmond A, Hu Q (Ed.) *Handbook of Microalgal Culture*. West Sussex, UK: John Wiley & Sons, Ltd, 545–565

Liang Y, Beardall J, Heraud P (2006) Effect of UV radiation on growth, chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae). *Phycologia*, 45: 605–615

Liolios K, Mavromatis K, Tavernarakis N, Kyrpides NC (2008) The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res*. 36(Database issue): D475-479

Lithwick G, Margalit H (2003) Hierarchy of sequence-dependent features associated with prokaryotic translation. *Genome Res.*, 13: 2665–2673

Liu B, Benning C (2013) Lipid metabolism in microalgae distinguishes itself. *Curr. Opin. Biotechnol*., 24(2): 300–309

Liu W, Yuan JS, Stewart Jr CN (2013) Advanced genetic tools for plant biotechnology. *Nat. Rev. Genet*., 14(11): 781–793

Livne A, Sukenik A (1990) Acetyl coenzyme A carboxylase from the marine prymnesiophyte *Isochrysis galbana. Plant Cell Physiol*., 31: 851–858

Lluisma AO, Ragan MA (1997) Expressed sequence tags (ESTs) from the marine red alga G*racilaria gracilis*. *J. Appl. Phycol.*, 9: 287–293

Lombardi AT, Wangersky PJ (1991) Influence of phosphorus and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures. *Mar. Ecol. Prog. Ser*., 77: 39–77

Lozano JC, Schatt P, Botebol H, Vergé V, Lesuisse E, Blain S, Carré IA, Bouget FY (2014), Efficient gene targeting and removal of foreign DNA by homologous recombination in the picoeukaryote Ostreococcus. *Plant J.*, 78: 1073–1083

Lumbreras V, Stevens DR, Purton S (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.*, 14(4): 441–447

Lung SC, Weselake RJ (2006) Diacylglycerol acyltransferase: A key mediator of plant triacylglycerol synthesis. *Lipids* 41: 1073–1088

Lüning K, Titlyanov EA, Titlyanova TV (1997) Diurnal and circadian periodicity of mitosis and growth in marine macroalgae. 3. The red alga *Porphyra umbilicalis. Eur. J. Phycol*., 32: 167–173

Madoka Y, Tomizawa KI, Mizoi J, Nishida I, Nagano Y, Sasaki Y (2002) Chloroplast transformation with modified accD operon increases acetyl-CoA carboxylase and causes extension of leaf longevity and increase in seed yield in tobacco. *Plant Cell Physiol*., 43: 1518–1525

Maheswari U, Montsant A, Goll J, Krishnasamy S, Rajyashri KR, Patell VM, Bowler C (2005) The Diatom EST Database. *Nucleic Acids Res*., 33(Database issue): D344-347

Maisonneuve S, Bessoule JJ, Lessire R, Delseny M, Roscoe TJ (2010) Expression of rapeseed microsomal lysophosphatidic acid acyltransferase Isozymes enhances seed oil content in Arabidopsis. *Plant Physiol*., 152: 670–684

Mann DG (1993) Patterns of sexual reproduction in diatoms. In: *Twelfth International Diatom Symposium*, (pp. 11-20). Springer Netherlands

Marín-Navarro J, Manuell AL, Wu J, P Mayfield S (2007) Chloroplast translation regulation. *Photosynth Res*., 94(2-3): 359-374

Martins DA, Custódio L, Barreira L, Pereira H, Ben-Hamadou R, Varela J, Abu-Salah KM (2013) Alternative sources of n-3 long-chain polyunsaturated fatty acids in marine microalgae. *Mar. Drugs.*, 11: 2259–2281

Maruyama M, Horáková I, Honda H, Xing X, Shiragami N, Unno H (1994) Introduction of foreign DNA into *Chlorella saccharophila* by electroporation. *Biotechnol. Tech*., 8: 821–826

Matsuzaki M, Misumi O, Shin-I T, Maruyama S, Takahara M, Miyagishima SY, Mori T, Nishida K, Yagisawa F, Nishida K, Yoshida Y, Nishimura Y, Nakao S, Kobayashi T, Momoyama Y, Higashiyama T, Minoda A, Sano M, Nomoto H, Oishi K, Hayashi H, Ohta F, Nishizaka S, Haga S, Miura S, Morishita T, Kabeya Y, Terasawa K, Suzuki Y, Ishii Y, Asakawa S, Takano H, Ohta N, Kuroiwa H, Tanaka K, Shimizu N, Sugano S, Sato N, Nozaki H, Ogasawara N, Kohara Y, Kuroiwa T (2004) Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature*, 6983: 653-657

Mayfield SP, Franklin SE, Lerner RA (2003) Expression and assembly of a fully active antibody in algae. *Proc. Natl. Acad. Sci. USA*, 100(2): 438–442

Mayfield SP, Schultz J (2004) Development of a luciferase reporter gene, luxCt, for *Chlamydomonas reinhardtii* chloroplast. *Plant J.*, 37(3): 449–458

Mendiola-Morgenthaler L, Eichenberger W, Boschetti A (1985) Isolation of chloroplast envelopes from Chlamydomonas. Lipid and polypeptide composition. *Plant Sci*., 41: 97–104

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L, Marshall WF, Qu LH, Nelson DR, Sanderfoot AA, Spalding MH, Kapitonov VV, Ren Q, Ferris P, Lindquist E, Shapiro H, Lucas SM, Grimwood J, Schmutz J, Cardol P, Cerutti H, Chanfreau G, Chen CL, Cognat V, Croft MT, Dent R, Dutcher S, Fernandez E, Fukuzawa H, Gonzalez-Ballester D, Gonzalez-Halphen D, Hallmann A, Hanikenne M, Hippler M, Inwood W, Jabbari K, Kalanon M, Kuras R, Lefebvre PA, Lemaire SD, Lobanov AV, Lohr M, Manuell A, Meier I, Mets L, Mittag M, Mittelmeier T, Moroney JV, Moseley J, Napoli C, Nedelcu AM, Niyogi K, Novoselov SV, Paulsen IT, Pazour G, Purton S, Ral JP, Riano-Pachon DM, Riekhof W, Rymarquis L,

Schroda M, Stern D, Umen J, Willows R, Wilson N, Zimmer SL, Allmer J, Balk J, Bisova K, Chen CJ, Elias M, Gendler K, Hauser C, Lamb MR, Ledford H, Long JC, Minagawa J, Page MD, Pan J, Pootakham W, Roje S, Rose A, Stahlberg E, Terauchi AM, Yang P, Ball S, Bowler C, Dieckmann CL, Gladyshev VN, Green P, Jorgensen R, Mayfield S, Mueller-Roeber B, Rajamani S, Sayre RT, Brokstein P, Dubchak I, Goodstein D, Hornick L, Huang YW, Jhaveri J, Luo Y, Martinez D, Ngau WC, Otillar B, Poliakov A, Porter A, Szajkowski L, Werner G, Zhou K, Grigoriev IV, Rokhsar DS, Grossman AR (2007) The Chlamydomonas genome reveals the evolution of key animal and plant functions. *Science* 318: 245–250

Merchant SS, Kropat J, Liu B, Shaw J, Warakanont J (2012) TAG, you're it! Chlamydomonas as a reference organism for understanding algal triacylglycerol accumulation. *Curr. Opin. Biotechnol*., 23: 352–363

Meyer A, Cirpus P, Ott C, Schlecker R, Zähringer U, Heinz E (2003) Biosynthesis of docosahexaenoic acid in *Euglena gracilis*: biochemical and molecular evidence for the involvement of a Delta4-fatty acyl group desaturase. *Biochemistry-US*, 42: 9779–9788

Meyer A, Kirsch H, Domergue F, Abbadi A, Sperling P, Bauer J, Cirpus P, Zank TK, Moreau H, Roscoe TJ, Zähringer U, Heinz E (2004) Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res.*, 45: 1899–1909

Mhaske V, Beldjilali K, Ohlrogge J, Pollard M (2005) Isolation and characterization of an *Arabidopsis thaliana* knockout line for phospholipid: diacylglycerol transacylase gene (At5g13640). *Plant Physiol. Biochem*., 43: 413–417

Michael R, Miller DJ 1998. Genetic transformation of dinoflagellates (Amphidinium and Symbiodinium): expression of GUS in microalgae using heterologous promoter constructs. *Plant J*., 13: 427–435

Michaelson LV, Lazarus CM, Griffiths G, Napier JA, Stobart AK (1998) Isolation of a Δ5-Fatty Acid Desaturase Gene from *Mortierella alpine*. *J. Biol. Chem.*, 273: 19055–59

Millar AA, Wrischer M, Kunst L (1998) Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. *The Plant Cell Online*, 10(11): 1889-1902

Miller R, Wu GX, Deshpande RR, Vieler A, Gartner K, Li XB, Moellering ER, Zauner S, Cornish AJ, Liu BS, Bullard B, Sears BB, Kuo MH, Hegg EL, Shachar-Hill Y, Shiu SH, Benning C

(2010) Changes in Transcript Abundance in *Chlamydomonas reinhardtii* following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiol.*, 154: 1737–1752

Minai L, Wostrikoff K, Wollman FA, Choquet Y (2006) Chloroplast biogenesis of photosystem II cores involves a series of assembly-controlled steps that regulate translations. *Plant Cell*, 18(1): 159–175

Minoda A, Sakagami R, Yagisawa F, Kuroiwa T, Tanaka K (2004) Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga, *Cyanidioschyzon merolae* 10D. *Plant Cell Physiol.*, 45: 667–671

Mitchel AG, Martin CE (1995) A Novel Cytochrome b5-like Domain Is Linked to the Carboxyl Terminus of the Saccharomyces cerevisiae Δ-9 Fatty Acid Desaturase. *J. Biol. Chem.*, 270: 29766-29772

Moellering ER, Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii. Eukaryot. Cell*, 9: 97–106

Moellering ER, Muthan B, Benning C (2010) Freezing tolerance in plants requires lipid remodelling at the outer chloroplast membrane. *Science*, 330: 226–228

Moffat CF, McGill AS, Hardy R, Anderson RS (1993) The production of fish oils enriched in polyunsaturated fatty acid containing triglycerides. *J. Am. Oil. Chem. Soc.*, 70: 133–138

Molina Grima E, Robles Medina A, Giménez Giménez A, Ibáñez González MJ (1996) Gram-scale purification of eicosapentaenoic acid (EPA, 20:5n-3) from wet *Phaeodactylum tricornutum* UTEX 640 biomass. *J. Appl. Phycol.*, 8: 359–367

Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D (2009) Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii. Plant J.*, 58(1): 165-174

Montsant A, Andrew EA, Coesel S, De Martino A, Falciatore A, Heijde M, Jabbari K, Maheswari U, Mangogna M, Rayko E (2007) Identification and comparative genomic analysis of signaling and regulatory mechanisms in the diatom *Thalassiosira pseudonana. J. Phycol*., 43: 585-604

Morel FMM, Rueter JG, Anderson DM, Guillard RRL (1979) Aquil: A chemically defined phytoplankton culture medium for trace metal studies. *J. Phycol*., 15: 135–141

Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. *Science*, 326(5959): 1501-1501

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H (2012) Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. *Phytochemistry*, 75: 50–59

Murai N, Sutton DW, Murray MG, Slightom JL, Merlo DJ, Reichert NA, Sengupta-Gopalan C, Stock CA, Barker RF, Kemp JD, Hall TC (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. *Science*, 222:476-482

Murphy DJ, Vance J (1999) Mechanisms of lipid-body formation. *Trends Biochem. Sci*., 24: 109–115

Murphy DJ (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog. Lipid Res*., 40: 325–438

Nagle CA, Vergnes L, Dejong H, Wang S, Lewin TM, Reue K, Coleman RA (2008) Identification of a novel sn-glycerol-3-phosphate acyltransferase isoform, GPAT4, as the enzyme deficient in Agpat6-/- mice. *J. Lipid Res*., 49: 823–831

Nakamura Y, Koizumi R, Shui GH, Shimojima M, Wenk MR, Ito T, Ohta H (2009) *Arabidopsis lipins* mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proc. Natl. Acad. Sci. USA*, 106: 20978–20983

Nakamura Y, Tsuchiya M, Ohta H (2007) Plastidic phosphatidic acid phosphatases identified in a distinct subfamily of lipid phosphate phosphatases with prokaryotic origin. *J. Biol. Chem*. 282: 29013–29021

Napier JA, Sayanova O, Qi BX, Lazarus CM (2004) Progress toward the production of long-chain polyunsaturated fatty acids in transgenic plants. *Lipids*, 39: 1067–1075

Napier JA (2007). The production of unusual fatty acids in transgenic plants. *Annu. Rev. Plant Biol*., 58: 295-319

Napier JA, Sayanova O, Hamilton M, Vaezi R (2014) Recombinant organisms. WO patent: WO 2014053821 A1

Nellen W, Lichtenstein C (1993) What makes an mRNA anti-sense-itive? *Trends Biochem. Sci*., 18: 419–423

Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.*, 1(7): 841–845

Nguyen HM, Baudet M, Cuine S, Adriano JM, Barthe D, Billon E, Bruley C, Beisson F, Peltier G, Ferro M, Li-Beisson Y (2011) Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii*: with focus on proteins involved in lipid metabolism. *Proteomics*, 11: 4266–4273

Nguyen RL, Tam LW, Lefebvre PA (2005) The LF1 gene of *Chlamydomonas reinhardtii* encodes a novel protein required for flagellar length control. *Genetics*, 169: 1415–1424

Nicol S, Foster J, Kawaguchi S (2012) The fishery for Antarctic krill—recent developments. *Fish Fish*, 13: 30–40

Nikaido I, Asamizu E, Nakajima M, Nakamura Y, Saga N, Tabata S (2000) Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga, *Porphyra yezoensis*. *DNA Res*., 7: 223-227

Niu Y, Kong J, Fu L, Yang J, Xu Y (2009) Identification of a Novel C20-elongase Gene from the Marine Microalgae *Pavlova viridis* and its Expression in *Escherichia coli. Marine Biotechnology*, 11 (1): 17–23

Niu YF, Zhang MH, Li DW, Yang WD, Liu JS, Bai W, Li HY (2013) Improvement of neutral lipid and polyunsaturated fatty acid biosynthesis by overexpressing a type 2 diacylglycerol acyltransferase in marine diatom *Phaeodactylum tricornutum*. *Mar. Drugs*., 11: 4558–4569

Noordally ZB, Millar AJ (2014) Clocks in algae. *Biochemistry*, 54 (2): 171–183

Novikoff AB, Novikoff PM, Rosen OM, Rubin CS (1980) Organelle relationships in cultured 3t3-L1 preadipocytes. *J. Cell Biol*. 87: 180–196

O'Brien EA, Koski LB, Zhang Y, Yang L, Wang E, Gray MW, Burger G, Lang BF (2007) TBestDB: a taxonomically broad database of expressed sequence tags (ESTs). *Nucleic Acids Res*., 35(Database issue): D445-451

Oelkers P, Tinkelenberg A, Erdeniz N, Cromley D, Billheimer JT, Sturley SL (2000) A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. *J. Biol. Chem*., 275: 15609–15612

Oey M, Ross IL, Hankamer B (2014) Gateway-Assisted Vector Construction to Facilitate Expression of Foreign Proteins in the Chloroplast of Single Celled Algae. *PLoS ONE*, 9(2): e86841. doi: 10.1371/journal.pone.0086841

Ohlrogge JB (1994) Desing of new plant products: engineering of fatty acid metabolism. *Plant Physiol.*, 104 (3): 821–826

Ohlrogge J, Browse J (1995) Lipid biosynthesis. *Plant Cell*, 7: 957–970

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 48: 109–136

Oo KC, Huang AHC (1989) Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. *Plant Physiol*., 91: 1288–1295

Pal D, Khozin-Goldberg I, Cohen Z, Boussiba S (2011) The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. *Appl Microbiol Biotechnol.*, 1–13

Palenik B, Grimwood J, Aerts A, Rouzé P, Salamov A, Putnam N, Dupont C, Jorgensen R, Derelle E, Rombauts S, Zhou K, Otillar R, Merchant SS, Podell S, Gaasterland T, Napoli C, Gendler K, Manuell A, Tai V, Vallon O, Piganeau G, Jancek S, Heijde M, Jabbari K, Bowler C, Lohr M, Robbens S, Werner G, Dubchak I, Pazour GJ, Ren Q, Paulsen I, Delwiche C, Schmutz J, Rokhsar D, Van de Peer Y, Moreau H, Grigoriev IV (2007) The tiny eukaryote Ostreococcus provides genomic insights into the paradox of plankton speciation. *Proc. Natl. Acad. Sci. USA*, 104: 7705-7710

Parsons TR, Stephens K, Trickland JDH (1961). On the chemical composition of eleven species of marine phytoplankters. *J. Fish Res. Board Can.*, 18(6), 1001-1016

Pereira SL, Leonard AE, Mukerji P (2003) Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins Leukot. Essent. Fatty Acids*, 68: 97–106

Pereira SL, Leonard AE, Huang YS, Chuang LT, Mukerji P (2004) Identification of two novel microalgal enzymes involved in the conversion of the omega3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid. *Biochem. J.*, 384: 357–366

Pérez-Rodríguez P, Riaño-Pachón DM, Corrêa LG, Rensing SA, Kersten B, Mueller-Roeber B (2010) PlnTFDB: updated content and new features of the plant transcription factor database. *Nucleic Acids Res.*, 38(Database issue): D822-827

Periappuram C, Steinhauer L, Barton DL, Taylor DC, Chatson B, Zou JT (2000) The plastidic phosphoglucomutase from Arabidopsis. A reversible enzyme reaction with an important role in metabolic control. *Plant Physiol.*, 122: 1193–1199

Pernet F, Tremblay R, Demers E, Roussy M (2003) Variation of lipid class and fatty acid composition of *Chaetoceros muelleri* and *Isochrysis* sp. grown in a semicontinuous system. *Aquaculture*, 221(1): 393-406

Peterfy M, Phan J, Xu P, Reue K (2001) Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat. Genet*., 27: 121–124

Petrie JR, Liu Q, Mackenzie AM, Shrestha P, Mansour MP, Robert SS, Frampton DF, Blackburn SI, Nichols PD, Singh SP (2009) Isolation and characterisation of a high-efficiency desaturase and elongases from microalgae for transgenic LC-PUFA production. *Mar. Biotechnol.*, 12: 430-438

Petrie JR, Shrestha P, Mansour MP, Nichols PD, Liu Q, Singh SP (2010) Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with ω3-preference from the marine microalga *Micromonas pusilla*. *Metab. Eng.*, 12: 233–240

Petrie JR, Shrestha P, Zhou XR, Mansour MP, Liu Q, Belide S, Nichols PD, Singh SP (2012) Metabolic Engineering Plant Seeds with Fish Oil-Like Levels of DHA. *PLoS One*, 7: e49165

Pfeuty B, Thommen Q, Corellou F, Djouani-Tahri EB, Bouget FY, Lefranc M (2012) Circadian clocks in changing weather and seasons: Lessons from the picoalga *Ostreococcus tauri. Bioessays*, 34: 781–790

Pidoux G, Witczak O, Jarnaess E, Myrvold L, Urlaub H, Stokka AJ, Kuntziger T, Tasken K (2011) Optic atrophy 1 is an A-kinase anchoring protein on lipid droplets that mediates adrenergic control of lipolysis. *Embo J.*, 30: 4371–4386

Piorreck M, Baasch KH, Pohl P (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry*, 23: 207–216

Piorreck M, Pohl P (1984) Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase. *Phytochemistry*, 23: 217–223

Postbeittenmiller D, Roughan G, Ohlrogge JB (1992) Regulation of plant fatty acid biosynthesis: analysis of acyl-coenzyme A and acyl-acyl carrier protein substrate pools in spinach and pea chloroplasts. *Plant Physiol.*, 100: 923–930

Potvin G, Zhang ZS (2010) Strategies for high-level recombinant protein expression in transgenic microalgae: a review. *Biotechnol. Adv.*, 28(6): 910–918

Poulsen N, Kröger N (2005) A new molecular tool for transgenic diatoms: control of mRNA and protein biosynthesis by an inducible promoter-terminator cassette. *FEBS J.*, 272(13): 3413-3423

Poulsen N, Chesley PM, Kröger N (2006) Molecular genetic manipulation of the diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.*, 42: 1059–1065

Price NM, Harrison GI, Hering JG, Hudson RJ, Nirel PMV, Palenik B, Morel FMM (1989) Preparation and chemistry of the artificial algal culture medium Aquil. *Biol. Oceanogr.*, 6: 443–461

Pruitt KD, Tatusova T, Maglott DR (2005) NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.*, 33(Database issue): D501-504

Qi B, Beaudoin F, Fraser T, Stobart AK, Napier JA, Lazarus CM (2002) Identification of a cDNA encoding a novel C18-Δ9 polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Lett.*, 510:159–65

Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, ButlerJ, Napier J, Stobart A, Lazarus C (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nat. Biotechnol.*, 22: 739–745

Qin S, Sun GQ, Jiang P, Zou LH, Wu Y, Tseng CK (1999) Review of genetic engineering of *Laminaria japonica* (Laminariales, Phaeophyta) in China. *Hydrobiologia*, 398: 469–472

Qin S, Yu DZ, Jiang P, Teng CY, Zeng CK (2003) Stable expression of lacZ reporter gene in seaweed *Undaria pinnatifida. High Technol. Lett*., 13: 87–89

Rabbani S, Beyer P, Von Lintig J, Hugueney P, Kleinig H (1998) Induced beta-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil. Plant Physiol*., 116: 1239–1248

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010) Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell*, 9(4): 486–501

Radakovits R, Eduafo P, Posewitz M (2011) Genetic engineering of fatty acid chain length in *Phaeodactylum tricornutum*. *Metab. Eng*., 13: 89–95

Radakovits R, Jinkerson RE, Fuerstenberg SI, Tae H, Settlage RE, Boore JL, Posewitz MC (2012) Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. *Nat Commun*., 3: 686

Raible F, Falciatore A (2014). It's about time: Rhythms as a new dimension of molecular marine research. *Mar. Genomics*, 14: 1-2

Reddy AS, Thomas TL (1996). Expression of a cyanobacterial Δ6-desaturase gene results in γ-linolenic acid production in transgenic plants. *Nat. Biotechnol*., 14:639–42

Rehstam-Holm AS, Godhe A (2003) Genetic Engineering of Algal Species, Eolss Publishers, Oxford, UK

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science*, 290: 2105–2110

Robert S, Singh SP, Zhou XR, Petrie JR, Blackburn SI, Mansour PM, Nichols PD, Liu Q, Green A (2005) Metabolic engineering of Arabidopsis to produce nutritionally important DHA in seed oil. *Funct. Plant Biol.*, 32: 473–479

Robert SS (2006) Production of eicosapentaenoic and docosahexaenoic acid-containing oils in transgenic land plants for human and aquaculture nutrition. *Mar. Biotechnol*., 8: 103–9

Robert SS, Petrie JR, Zhou XR, Mansour MP, Blackburn SI, Green AG, Singh SP, Nichols PD (2008) Isolation and Characterisation of a Δ5-fatty Acid Elongase from the Marine Microalga *Pavlova salina*. *Marine Biotechnology*, 11 (3): 410–418

Robert SS, Petrie JR, Zhou XR, Mansour MP, Blackburn SI, Green AG, Singh SP, Nichols PD (2009) Isolation and characterisation of a delta5-fatty acid elongase from the marine microalga *Pavlova salina*. *Mar. Biotechnol*., 11, pp. 410–418

Roessler PG (1988) Effects of silicon deficiency on lipid composition and metabolism in the Diatom *Cyclotella cryptica. J. Phycol*., 24: 394–400

Roessler PG (1990) Purification and characterization of acetyl-CoA carboxylase from the diatom *Cyclotella cryptica. Plant Physiol*., 92: 73–78

Roessler PG, Ohlrogge JB (1993) Cloning and characterization of the gene that encodes acetyl coenzyme A carboxylase in the alga *Cyclotella cryptica. J. Biol. Chem*. 268: 19254–19259

Rothamsted Research (2014) GM Camelina field Trial: Public information [Online] [Accessed July 2014] Available from: http://www.rothamsted.ac.uk/camelina

Round FE, Crawford RM, Mann DG (2007) THE DIATOMS: Biology and Morphology of the Genera. *Cambridge University Press*

Rubio-Rodríguez N, de-Diego-Rupérez S, Beltrán S, Jaime I Sanz MT, Rovira J (2008) Supercritical fluid extraction of the omega-3 rich oil contained in hake (*Merluccius capensis–Merluccius paradoxus*) byproducts: study of the influence of process parameters on the extraction yield and oil quality. *J. Supercrit.l Fl.*, 47: 215–226

Rubio-Rodríguez N, de Diego SM, Beltrán S, Jaime I, Sanz MT, Rovira J (2012) Supercritical fluid extraction of fish oil from fish by-products: A comparison with other extraction methods. *J. Food Eng.*, 109 (2): 238-248

Ruiz-López N, Haslam RP, Venegas-Calerón M, Li T, Bauer J, Napier JA, Sayanova O (2012) Enhancing the accumulation of omega-3 long chain polyunsaturated fatty acids in transgenic *Arabidopsis thaliana* via iterative metabolic engineering and genetic crossing. *Transgenic Res.*, 21: 1233–1243

Ruiz-Lopez N, Haslam RP, Napier JA, Sayanova O (2013) Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. *Plant J.*, 77: 198-208

Ruiz-Lopez N, Haslam RP, Usher SL, Napier JA, Sayanova O (2013) Reconstitution of EPA and DHA biosynthesis in Arabidopsis: Iterative metabolic engineering for the synthesis of n-3 LC-PUFAs in transgenic plants. *Metab. Eng*., 17: 30–41

Ruuska SA, Girke T, Benning C, Ohlrogge JB (2002) Contrapuntal networks of gene expression during Arabidopsis seed filling. *Plant Cell*, 14: 1191–1206

Saha S, Enugutti B, Rajakumari S, Rajasekharan R (2006) Cytosolic triacylglycerol biosynthetic pathway in oilseeds. Molecular cloning and expression of peanut cytosolic diacylglycerol acyltransferase. *Plant Physiol*.141: 1533–1543

Sakurai K, Mori N, Sato N (2014) Detection and characterization of phosphatidylcholine in various strains of the genus Chlamydomonas (Volvocales, Chlorophyceae). *J Plant Res*., 127(5): 641-650

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*

Saravanan P, Davidson NC, Schmidt EB, Calder PC (2010) Cardiovascular effects of marine omega-3 fatty acids. *Lancet*, 375: 540–550

Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: Structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Biosci. Biotechnol. Biochem*., 68: 1175–1184

Sato S, Xing AQ, Ye XG, Schweiger B, Kinney A, Graefc G, Clemente T (2004) Production of γ-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. *Crop Sci*., 44:646–52

Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA (1997) Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of Δ6-desaturated fatty acids in transgenic tobacco. *Proc. Natl. Acad. Sci. USA*, 94: 4211–4216

Sayanova O, Napier JA, Shewry PA (1999) Δ6-Unsaturated fatty acids in species and tissues of the Primulaceae. *Phytochemistry*, 52: 419–422

Sayanova O, Beaudoin F, Michaelson LV, Shewry PR, Napier JA (2003) Identification of Primula fatty acid Δ6-desaturases with n-3 substrate preferences. *FEBS Lett*., 542: 100–104

Sayanova O, Napier JA (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. *Phytochemistry*, 65:147–58

Sayanova O, Haslam R, Qi BX, Lazarus CM, Napier JA (2006) The alternative pathway C20 Δ8-desaturase from the nonphoto synthetic organism *Acanthamoeba castellanii* is an atypical cytochrome b5-fusion desaturase. *FEBS Lett.*, 580:1946–52

Sayanova O, Haslam R, Venegas-Calerón M, Napier JA (2006) Identification of Primula "front-end" desaturases with distinct n-6 or n-3 substrate preferences. *Planta*, 224: 1269–1277

Sayanova O, Haslam R, Venegas-Calerón M, Ruiz-Lopez N, Worthy C, Rooks P, Allen MJ, Napier JA (2011) Identification and functional characterisation of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore *Emiliania huxleyi. Phytochemistry*, 72: 594–600

Sayanova O, Ruiz-Lopez N, Haslam RP, Napier JA (2012) The role of Δ6-desaturase acyl-carrier specificity in the efficient synthesis of long-chain polyunsaturated fatty acids in plants. *Plant Biotechnol. J.*, 10: 195–206

Scala S, Carels N, Falciatore A, Chiusano ML, Bowler C (2002) Genome properties of the diatom *Phaeodactylum tricornutum*. *Plant Physiol*., 129: 993-1002

Schell J, Van Montagu M (1977) The Ti-Plasmid of *Agrobacterium Tumefaciens*, A Natural Vector for the Introduction of NIF Genes in Plants?. In: Hollaender A, Burris RH, Day PR, Hardy RWF, Helinski DR, Lamborg MR, Owens L, Valentine RC (Ed.) *Genetic Engineering for Nitrogen Fixation - Basic Life Sciences*, 9: 159-179

Schenk PM, Hall SRT, Stephens E, Marx UC, Mussgnug JH, Posten C, Kruse O, Hankamer B (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenerg. Res.*, 1: 20–43

Schiedlmeier B, Schmitt R, Müller W, Kirk MM, Gruber H, Mages W, Kirk DL (1994) Nuclear transformation of *Volvox carteri*. *Proc. Natl. Acad. Sci. USA*, 91: 5080–5084

Schuhmann H, Lim DKY, Schenk PM (2012) Perspectives on metabolic engineering for increased lipid contents in microalgae. *Biofuels*, 3: 71–86

Sheehan J, Dunahay T, Benemann J, Roessler P (1998) A look back at the US Department of Energy's Aquatic Species Program—biodiesel from algae. Report no. NREL/TP-580-24190 National Renewable Energy Laboratory, Golden, Colorado

Shestakov SV, Khyen NT (1970) Evidence for genetic transformation in blue-green alga *Anacystis nidulans*. *Molec. Gen. Genet*., 107: 372–375

Shifrin NS, Chisholm SW (1981) Phytoplancton lipids: interspecific differences and effects of nitrate, silicate and light–dark cycles. *J. Phycol*., 17: 374–384

Shimko N, Liu L, Lang BF, Burger G (2001) GOBASE: the organelle genome database. *Nucleic Acids Res*., 29: 128-132

Shimogawara K, Fujiwara S, Grossman A, Usuda H (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, 148: 1821–1828

Shockey JM, Gidda SK, Chapital DC, Kuan JC, Dhanoa PK, Bland JM, Rothstein SJ, Mullen RT, Dyer JM (2006) Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. *Plant Cell*, 18: 2294–2313

Shrager J, Hauser C, Chang C W, Harris EH, Davies J, McDermott J, Tamse R, Zhang Z, Grossman AR (2003) *Chlamydomonas reinhardtii* genome project. A guide to the generation and use of the cDNA information. *Plant Physiol*., 131: 401–408

Sims PA, Mann DG, Medlin LK (2006) Evolution of the diatoms: insights from fossil, biological and molecular data. *Phycologia* 45: 361–402

Singh S, Zhou XR, Liu Q, Stymne S, Green AG (2005) Metabolic engineering of new fatty acids in plants. *Curr. Opin. Plant Biol*., 8: 197–203

Sizova I, Fuhrmann M, Hegemann P (2001) A Streptomyces rimosus aphVIII gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii. Gene*, 277(1-2): 221-229

Sizova I, Greiner A, Awasthi M, Kateriya S, Hegemann P (2013) Nuclear gene targeting in Chlamydomonas using engineered zinc-finger nucleases. *Plant J.*, 73(5): 873–882

Smith JL, Sherman DH (2008) Biochemistry – an enzyme assembly line. *Science*, 321: 1304–1305

Spencer AK, Greenspan AD, Cronan JE Jr (1978) Thioesterases I and II of *Escherichia coli*: hydrolysis of native acyl-acyl carrier protein thioesters. *J. Bio. Chem*., 253: 5922-5926

Stahl U, Carlsson AS, Lenman M, Dahlqvist A, Huang BQ, Banas, W., Banas, A. & Stymne, S. (2004) Cloning and functional characterization of a phospholipid: diacylglycerol acyltransferase from Arabidopsis. *Plant Physiol*., 135: 1324–1335

Stark A , Lin MF, Kheradpour P, Pedersen JS, Parts L, Carlson JW, Crosby MA, Rasmussen MD, Roy S, Deoras AN, Ruby JG, Brennecke J, Hodges E, Hinrichs AS, Caspi A, Park SW, Han MV, Maeder ML, Polansky BJ, Robson BE, Aerts S, van Helden J, Hassan B, Gilbert DG, Eastman DA, Rice M, Weir M, Hahn MW, Park Y, Dewey CN, Pachter L, Kent WJ, Haussler D, Lai EC, Bartel DP, Hannon GJ,; Kaufman TC, Eisen MB, Clark AG, Smith D, Celniker SE, Gelbart WM, Kellis M (2007) Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. *Nature* 450: 219–232

Sukenik A, Wahnon R (1991) Biochemical quality of marine unicellular algae with special emphasis on lipid composition: I. *Isochrysis galbana. Aquaculture*, 97: 61–72

Sukenik A, Yamaguchi Y, Livne A (1993) Alterations in lipid molecular-species of the marine eustigmatophyte *Nannochloropsis* sp. *J. Phycol*., 29: 620–626

Sun Y, Yang Z, Gao X, Li Q, Zhang Q, Xu Z (2005) Expression of foreign genes in Dunaliella by electroporation. *Mol. Biotechnol*., 30: 185–192

Sun Y, Gao X, Li Q, Zhang Q, Xu Z (2006) Functional complementation of a nitrate reductase defective mutant of a green alga *Dunaliella viridis* by introducing the nitrate reductase gene. *Gene*, 377: 140–149

Sunda WG, Price NM, Morel FMM (2005) Trace metal ion buffers and their use in culture studies. In: *Algal Culturing Techniques*. Anderson, R.A., (Ed.) Academic Press: Amsterdam, The Netherlands, 35–63

Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, Rochaix JD, Ajam T, Surzycki S (2009) Factors effecting expression of vaccines in microalgae. *Biologicals*, 37(3): 133–138

Taguchi S, Hirata JA, Laws EA (1987) Silicate deficiency and lipid synthesis of marine diatoms. *J. Phycol*., 23: 260–267

Takagi M (2006) Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *J. Biosci. Bioeng.*, 101: 223–226

Tan C, Qin S, Zhang Q, Jiang P, Zhao F (2005) Establishment of a micro-particle bombardment transformation system for *Dunaliella salina. J. Microbiol*., 43: 361–365

Tang DK, Qiao SY, Wu M (1995) Insertion mutagenesis of *Chlamydomonas reinhardtii* by electroporation and heterologous DNA. *Biochem. Mol. Biol. Int*., 36(5): 1025-1035

Tardif M, Atteia A, Specht M, Cogne G, Rolland N, Brugière S, Hippler M, Ferro M, Bruley C, Peltier G, Vallon O, Cournac L (2012) PredAlgo, a new subcellular localization prediction tool dedicated to green algae. *Mol. Biol. Evol*., 29(12): 3625-3639

Teng C, Qin S, Liu J, Yu D, Liang C, Tseng C (2002) Transient expression of lacZ in bombarded unicellular green alga *Haematococcus pluvialis. J. Appl. Phycol*. 14: 497–500

Tessmar-Raible K, Raible F, Arboleda E (2011), Another place, another timer: Marine species and the rhythms of life. *Bioessays*, 33: 165–172

Thompson PA (1992) Effect of variation in temperature: II. On the fatty acid composition of eight species of marine phytoplankton. *J. Phycol*., 28: 488–497

Thompson PA, Guo M (1992) Effect of variation in temperature: I. On the biochemical composition of eight species of marine phytoplankton. *J. Phycol*., 28: 481–488

Tonon T, Harvey D, Larson TR, Graham IA (2002) Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae. *Phytochemistry*, 61(1): 15-24

Tonon T, Harvey D, Qing R, Li Y, Larson TR, Graham IA (2004) Identification of a fatty acid Delta11-desaturase from the microalga *Thalassiosira pseudonana*. *FEBS Lett*., 563: 28–34

Tonon T, Sayanova O, Michaelson LV, Qing R, Harvey D, Larson TR, Li Y, Napier JA, Graham IA (2005a) Fatty acid desaturases from the microalga *Thalassiosira pseudonana. FEBS J.*, 272: 3401–3412

Tonon T, Qing R, Harvey D, Li Y, Larson TR, Graham IA (2005b). Identification of a long-chain polyunsaturated fatty acid acyl-coenzyme A synthetase from the diatom *Thalassiosira pseudonana*. *Plant Physiol.*, 138(1): 402-408

Tornabene TG, Holzer G, Lien S, Burris N (1983) Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. *Enzyme Microb. Technol*., 5: 435–440

Traitler T, Winter H, Richli H, Ingenbleek Y (1984) Characterization of gamma-linolenic acid in Ribes seed. *Lipids*, 19: 923–928

Trentacoste EM, Shrestha RP, Smith SR, Glé C, Hartmann AC, Hildebrand M, Gerwick WH (2013) Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth. *Proc Natl Acad Sci USA*, 110: 19748–19753

Tucker G (2003) Nutritional enhancement of plants. *Curr. Opin. Plant Biol*., 14: 221–25

Ucciani E (1995) Potential sources of gamma-linolenic acid. *Oleagineux corps gras lipides*, 2: 319–322

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.,* 11(9): 636-646

Urzica EI, Vieler A, Hong-Hermesdorf A, Page MD, Casero D, Gallaher SD, Kropat J, Pellegrini M, Benning C, Merchant SS (2013) Remodeling of membrane lipids in iron-starved *Chlamydomonas. J Biol Chem*, 288: 30246–30258

Vaezi R, Napier JA, Sayanova O (2013) Identification and functional characterization of genes encoding omega-3 polyunsaturated fatty acid biosynthetic activities from unicellular microalgae. *Mar Drugs*, 11: 5116–5129

van der Krol AR, Mol JN, Stuitje AR (1988) Antisense genes in plants: an overview. *Gene*, 72: 45–50

Van Wagenen J, Miller TW, Hobbs S, Hook P, Crowe B, Huesemann M (2012) Effects of light and temperature on fatty acid production in *Nannochloropsis salina*. *Energies*, 5: 731–740

Vechtel B, Eichenberger W, Ruppel G (1992) Lipid bodies in Eremosphaera viridis De Bary (Chlorophyceae). *Plant Cell Physiol.*, 33: 41–48

Venegas-Calerón M, Sayanova O, Napier JA (2009) An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. *Prog Lipid Res.*, 49: 108–119

Vieler A, Brubaker SB, Vick B, Benning C (2012) A lipid droplet protein of Nannochloropsis with functions partially analogous to plant oleosins. *Plant Physiol*., 158: 1562–1569

Vieler A, Wu G, Tsai CH, Bullard B, Cornish AJ, Harvey C, Reca IB, Thornburg C, Achawanantakun R, Buehl CJ, Campbell MS, Cavalier D, Childs KL, Clark TJ, Deshpande R, Erickson E, Armenia Ferguson A, Handee W, Kong Q, Li X, Liu B, Lundback S, Peng C, Roston RL, Sanjaya, Simpson JP, Terbush A, Warakanont J, Zäuner S, Farre EM, Hegg EL, Jiang N, Kuo MH, Lu Y, Niyogi KK, Ohlrogge J, Osteryoung KW, Shachar-Hill Y, Sears BB, Sun Y, Takahashi H, Yandell M, Shiu SH, Benning C (2012) Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis* sp.CCMP1779. *PLoS Genet*., 8:e1003064. doi: 10.1371/journal.pgen.1003064

Vigeolas H, Mohlmann T, Martini N, Neuhaus HE, Geigenberger P (2004) Embryo-specific reduction of ADP-Glc pyrophosphorylase leads to an inhibition of starch synthesis and a delay in oil accumulation in developing seeds of oilseed rape. *Plant Physiol*., 136: 2676–2686

Voigt RG, Jensen CL, Fraley JK, Rozelle JC, Brown FR, Heird WC (2000) Relationship between omega-3 long-chain polyunsaturated fatty acid status during early infancy and neurodevelopmental status at 1 year of age. *J. Hum. Nutr. Diet*., 15: 111–120

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell*, 136: 669–687

Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *J. Exp. Mar. Biol. Ecol.*, 128(3): 219-240

Volkman JK, Smith DJ, Eglinton G, Forsberg TEV, Corner EDS (1981). Sterol and fatty acid composition of four marine Haptophycean algae. *J. Mar. Biol. Assoc. UK*, 61(02), 509-527

Voss A, Reinhart M, Sankarappa S, Sprecher H (1991) The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *J. Biol. Chem.*, 266: 19995–20000

Wagner D, Przybyla D, Camp ROD, Kim C, Landgraf F, Lee KP, Wursch M, Laloi C, Nater M, Hideg E, Apel K (2004) The genetic basis of singlet oxygen induced stress responses of *Arabidopsis thaliana. Science*, 306: 1183–1185

Wagner M, Hoppe K, Czabany T, Heilmann M, Daum G, Feussner I, Fulda M (2010) Identification and characterisation of an acyl-CoA: Diacylglycerol acyltransferase 2 (DGAT2) gene from the microalga *O. tauri. Plant Phys. Biochem.*, 48: 407–416

Wahlund TM, Hadaegh AR, Clark R, Nguyen B, Fanelli M, Read BA (2004) Analysis of expressed sequence tags from calcifying cells of marine coccolithophorid (*Emiliania huxleyi*). *Mar Biotechnol*., 6, 278-290

Walsh PS, Metzger DA, Higuchi R (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, 10(4): 506-513

Walther TC, Farese RV (2009) The life of lipid droplets. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids*, 1791: 459–466

Wang D, Ning K, Li J, Hu J, Han D, Wang H, Zeng X, Jing X, Zhou Q, Su X, Chang X, Wang A, Wang W, Jia J, Wei L, Xin Y, Qiao Y, Huang R, Chen J, Han B, Yoon K, Hill RT, Zohar Y, Chen F, Hu Q, Xu J (2014) Nannochloropsis Genomes Reveal Evolution of Microalgal Oleaginous Traits. *PLoS Genet*., 10(1): e1004094. doi: 10.1371/journal.pgen.1004094

Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U (2009) Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starch-less *Chlamydomonas reinhardtii. Eukaryot. Cell*, 8: 1856–1868

Weber AP, Oesterhelt C, Gross W, Bräutigam A, Imboden LA, Krassovskaya I, Linka N, Truchina J, Schneidereit J, Voll H, Voll LM, Zimmermann M, Jamai A, Riekhof WR, Yu B, Garavito RM, Benning C (2004) EST-analysis of the thermo-acidophilic red microalga *Galdieria sulphuraria* reveals potential for lipid A biosynthesis and unveils the pathway of carbon export from rhodoplasts. *Plant Mol. Biol*., 55(1), 17-32

Weeks DP (2011) Homologous recombination in Nannochloropsis: A powerful tool in an industrially relevant alga. *Proc. Natl. Acad. Sci. USA*, 108: 20859–20860

Wendel AA, Lewin TM, Coleman RA (2009) Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. *Biochim. Biophys. Acta*, 1791: 501–506

Weyman PD, Beeri K, Lefebvre SC, Rivera J, McCarthy JK, Heuberger AL, Peers G, Allen AE, Dupont CL (2014) Inactivation of *Phaeodactylum tricornutum* urease gene using transcription activator-like effector nuclease-based targeted mutagenesis. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12254

Worden AZ, Lee JH, Mock T, Rouzé P, Simmons MP, Aerts AL, Allen AE, Cuvelier ML, Derelle E, Everett MV, Foulon E, Grimwood J, Gundlach H, Henrissat B, Napoli C, McDonald SM, Parker MS, Rombauts S, Salamov A, Von Dassow P, Badger JH, Coutinho PM, Demir E, Dubchak I, Gentemann C, Eikrem W, Gready JE, John U, Lanier W, Lindquist EA, Lucas S, Mayer KF, Moreau H, Not F, Otillar R, Panaud O, Pangilinan J, Paulsen I, Piegu B, Poliakov A, Robbens S, Schmutz J, Toulza E, Wyss T, Zelensky A, Zhou K, Armbrust EV, Bhattacharya D, Goodenough UW, Van de Peer Y, Grigoriev IV (2009) Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes Micromonas. *Science*, 324: 268-272

Wostrikoff K, Girard-Bascou J, Wollman FA, Choquet Y (2004) Biogenesis of PSI involves a cascade of translational autoregulation in the chloroplast of Chlamydomonas. *EMBO J*., 23(13): 2696–2705

Wyman C, Kanaar R (2006) DNA double-strand break repair: all's well that ends well. *Annu. Rev. Genet.*, 40: 363-383

Xie WH, Zhu CC, Zhang NS, Li DW, Yang WD, Liu JS, Li HY (2014) Construction of novel chloroplast expression vector and development of high efficient and economical transformation system for diatom *Phaeodactylum tricornutum. Mar. Biotechnol*., 16(5): 538–546

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science*, 287-(5451): 303–305

Yoon KS, Han DX, Li YT, Sommerfeld M, Hu Q (2012) Phospholipid:diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. *Plant Cell*, 24: 3708–3724

Yuan L, Knauf VC (1997) Modification of plant components. *Curr. Opin. Biotechnol*., 8: 227–233

Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, Van Montagu M, Goodman HM (1980) Tumor DNA structure in plant cells transformed by *A. tumefaciens*. *Science*, 209: 1385-1391

Zambryski P, Depicker A, Kruger K, Goodman HM (1982) Tumor induction of *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J. Mol. Appl. Genet.*, 1: 361-370

Zaslavskaia LA, Lippmeier JC, Kroth PG, Grossman AR, Apt KE (2000) Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. *J. Phycol.*, 36: 379–386

Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE (2001) Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. *Science*, 292: 2073–2075

Zhang H, Jin J, Tang L, Zhao Y, Gu X, Gao G, Luo J (2011) PlantTFDB 2.0: update and improvement of the comprehensive plant transcription factor database. *Nucleic Acids Res*., 39(Database issue): D1114-1117

Zhang M, Fan JL, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. *Plant Cell*, 21: 3885–3901

Zhang R, Patena W, Armbruster U, Gang SS, Blum SR, Jonikas MC (2014) High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. *Plant Cell*, 26(4): 1398-1409

Zhao T, Wang W, Bai X, Qi Y (2009) Gene silencing by artificial microRNAs in Chlamydomonas. *Plant J.*, 58(1): 157-164

Zhekisheva M, Boussiba S, Khozin-Goldberg I, Zarka A, Cohen Z (2002) Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. *J. Phycol*., 38: 325–331

Zheng Z, Xia Q, Dauk M, Shen W, Selvaraj G, Zou J (2003) Arabidopsis AtGPAT1, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility. *Plant Cell*, 15: 1872–1887

Zhou XR, Robert SS, Petrie JR, Frampton DM, Mansour MP, Blackburn SI, Nichols PD, Green AG, Singh SP (2007) Isolation and characterization of genes from the marine microalga *Pavlova salina* encoding three front-end desaturases involved in docosahexaenoic acid biosynthesis. *Phytochemistry*, 68: 785–796

Zhuan C, Hong G, Ming L, Peng J (2006) Engineering the acetyl-CoA transportation system of *Candida tropicalis* enhances the production of dicarboxylic acid. *Biotechnol. J*., 1: 68–74

Zorin B, Lu Y, Sizova I, Hegemann P (2009) Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the PHOT gene. *Gene*, 432(1-2): 91-96

Zou JT, Katavic V, Giblin EM, Barton DL, MacKenzie SL, Keller WA, Hu X, Taylor DC (1997) Modification of seed oil content and acyl composition in the brassicaceae by expression of a yeast sn-2 acyltransferase gene. *Plant Cell*, 9: 909–923

Zweytick D, Athenstaedt K, Daum G (2000) Intracellular lipid particles of eukaryotic cells. *Biochim. Biophys. Acta, Rev. Biomembr.*, 1469: 101–120