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Development of microalgae tools and techniques: metabolic engineering of lipid profiles

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in

Biology

by

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TABLE OF CONTENTS

Signature Page…….. iii

Table of Contents…….. iv

List of Abbreviations……... v

List of Figures…….. vi

List of Tables…….. vii

Acknowledgements…….. viii

Vita……... x

Abstract of the Dissertation……... xi

Introduction…….. 1

Chapter 1: Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae……... 6

Chapter 2: Rapid estimation of lipid, protein and dry weight in microalgae using a portable LED fluorometer…….. 19

Chapter 3: Metabolic engineering of *Chlamydomonas reinhardtii* for the production of Eicosadienoic and Eicosatrienoic acid through heterologous expression of a Δ9 elongase…... 25

Chapter 4: Molecular tools for transformation and characterization of *Chlorella*…….. 42

Conclusion…….. 56

LIST OF ABBREVIATIONS

TAG – triacylglycerol

PE – phosphatidyl ethanolamine

GCMS – gas chromatography mass spectrometry

HSM – high salt media

TLC – thin layer chromatography

ER – endoplasmic reticulum

RBCS2 – rubisco small subunit

TAP – tris acetate phosphate

UTR – untranslated region

LIST OF FIGURES

Figure 1.1 The general scheme of plant lipid biosynthetic pathway and representative chemical structures of petroleum diesel, biodiesel, and ethanol……..…...……. 9

Figure 2.1 Fluorescence-dry weight correlations for *Chlamydomonas reinhardtii* and *Scenedesmus dimorphus*……..……..... 21

Figure 2.2 Total lipid measured by BODIPY 495/505 fluorescence……... 23

Figure 3.1 Construct design and transformation……...…...…… 32

Figure 3.2 Fluorescence microscopy and western blot of mCherry:IG..……. 33

Figure 3.3 GC-MS……..…….…... 34

Figure 3.4 Transcript and lipid abundance of selected transformants…….....……... 35

Figure 3.5 iTRAQ quantification of elongase protein accumulation…….……. 35

Figure 3.6 Wild-type and IG16 transformant lipid profile by GCMS……...…….…. 36

Figure 3.7 Localization of C20 after nitrogen deprivation and refeeding……...… 37

Figure 4.1 Growth of various *Chlorella* species in TAP media……... 45

Figure 4.2 Cell wall digestion assay…...……...……. 52

LIST OF TABLES

Table 1.1 A list of genetic modifications to higher plants and their resulting changes in fatty acid content……..……. 12

Table 4.1 Peak culture density and biomass production for *Chlorella* species……..... 47

Table 4.2 Transformation conditions tested…… 50

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ABSTRACT OF THE DISSERTATION

Development of microalgae tools and techniques: metabolic engineering of lipid profiles

by

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Professor Stephen P. Mayfield, Chair

Microalgae are drawing increased attention from a variety of fields ranging from nutrition and health care to energy, and deservedly so. The potential of algae is almost unparalleled as a biomanufacturing platform. Microalgae can be used to produce complex human antibodies to target cancerous cells, or as crops for the production of high quality food, or even as sources of lipids for conversion into biofuels. They can grow to tremendous density in bioreactors, or be cultivated in open ponds where their yield per acre dominates that of higher plants.

Research in microalgae needs to take it to the next step, to transform potential into reality and make production strains of microalgae into designer products that are custom tailored to suit the needs of the industry. The diversity of microalgae is enormous, and as a result, it is unlikely that the strains we have identified today will be the same we see as ideal production strains tomorrow. So in order to continue advancing the field, techniques for ideal cultivation, genetic modification, and metabolic engineering will need to be developed. This thesis work seeks to do just that by covering cultivation technology, metabolic engineering of lipids, and genetic modification of potential production strains.

From a molecular perspective, a broad review of the current state of lipid metabolic engineering in both microalgae and higher plants is provided, covering both the biofuel relevant molecules as well as nutritionally relevant omega-3 fatty acids. New techniques for rapid interrogation of protein, lipid, and dry weight content in microalgae under either indoor or outdoor cultivation techniques are also described herein. These will enable more efficient harvesting and culturing techniques as well as more efficient use of fertilizers and nutrients in cultivation of microalgae. Proof of concept for custom tailoring lipid profile is demonstrated in the manipulation of *Chlamydomonas reinhardtii* lipid metabolism for the production of very long chain polyunsaturated fatty acids, which also provides insights into how changes in lipid profile can have unexpected effects and yield new insights on lipid metabolism. Finally, attempts to characterize and transform a variety of potential production species are covered and reveal challenges to the transformation of new microalgae species. Taken together, this work represents significant advances in the field with regard to both cultivation and transformation and metabolic engineering of microalgal species.

INTRODUCTION

As our society continues to grow, the stress placed on our environment increases as a result of our need to produce food and fuel to sustain the population. Biotechnology provides a means of ameliorating the pressure on our feedstocks and a potential source for renewable fuels in the future. Critical to accomplishing both of these goals will be metabolic engineering of lipid profiles.

Lipids play important roles in human health and nutrition and also serve as sources of fungible fuels to replace our reliance on petroleum. As oil stocks continue to decline, prices for fuel rise and the need for a renewable alternative grows stronger. Because producing food requires the use of fossil fuels, the inevitable decline of petroleum availability will result in increases in the price of food. Thus, the production of both food and energy are inextricably linked. Without a means of generating a renewable source of energy and more efficient production of nutritive biomass for the world's masses, our society is doomed to catastrophic failure.

Higher plants have long been studied and used as testing ground for the development of molecular biology, and numerous techniques and tools have been developed for transforming their genomes. These genetic modifications have allowed scientists not only to study the fundamental biology of many plants but also to create directed changes in them, in order to create more productive plants or to alter their nutritional value. More productive strains of common food crops such as corn, rice, and tomatoes will be important for improving agricultural efficiency in land use, which will become a problem as urban sprawl continues.

Single celled algae called microalgae present an interesting solution to the problems of overpopulation and energy shortage. Microalgae are capable of incredibly high land use efficiency, with yields per acre much higher than traditional terrestrial crops. Additionally, microalgae are an incredibly diverse group of organisms with the potential to produce many valuable lipids and nutrients which will be crucial in either a food setting or for use as fuel. Although the field of microalgae genetic engineering is not as well developed as that of terrestrial plants, there is significant potential for transformative technology to be developed.

Commercial interest in microalgae research has been scaled up recently as a result of the great potential for productivity and biomanufacturing shown by species of microalgae. The most commonly studied and the most well characterized microalgae is *Chlamydomonas reinhardtii,* often referred to as "the green yeast". Although it is genetically tractable, there are still significant advances yet to be made in order to truly make *Chlamydomonas* or any other microalgae an industrially relevant producer.

Chapter 1 provides a broad overview of the state of metabolic engineering of lipid metabolism in higher plants as well as microalgae. Genetic engineering and metabolic engineering are more advanced in higher plants and can serve as a template for which modifications can be successful when applied to microalgae. Because of their ability to vastly outproduce terrestrial plants, microalgae are looked upon as food and fuel sources for the future. As a result of their broad and diverse lipid metabolism, microalgae are potential sources of smaller chain fatty acids for use in fuel and for conversion into fungible fuels but are also investigated for their ability to produce the very long chain polyunsaturated fatty acids which play important roles in human health and nutrition. Modifications that can boost productivity of lipids per gram of biomass will be very important to both applications because of the need to maximize nutrient utilization and efficiency of biomass conversion. Importantly, these modifications are not solely discussed for the model organism *Chlamydomonas,* but also for a number of other microalgae which have been identified as genetically tractable or high value strains due to their robust growth or productivity.

Chapter 2 concerns the development of rapid diagnostic tools for microalgae cultivation. To date, most of the cultivation techniques for microalgae rely on educated guesses or alternatively laborious and slow techniques to assess the potential yields and ideal harvest times for algae cultures. The goal of this research was to enable assessment of the primary products of microalgae cultivation in a rapid manner. Algae are primarily cultivated for their lipids, protein, and total biomass so techniques which allowed these to be rapidly assessed were investigated. These

techniques were developed for use in both laboratory strains and likely production strains of microalgae, and were tested in both small and large scale cultivation conditions. Enabling rapid assessment of the productivity of algae cultures is important for optimal harvesting but also in order to reduce the use of nutrients such as nitrogen in the cultivation of microalgae. Nitrogen, potassium and phosphorus are the key macro nutrients of fertilizer and currently require huge amounts of energy from petroleum sources to obtain. By using advanced screening techniques, farmers can time their additions of nutrients to maximize utilization and crop yield and avoid using excess fertilizer as is the case with current agricultural practice. Not only will this make microalgae farming more environmentally friendly, it will also serve to reduce the cost of production. Fluorescence based sample interrogation is an avenue of research that has yielded excellent results in molecular biology and now will begin to be utilized even in a non-laboratory setting to improve cultivation efficiency.

In Chapter 3, metabolic engineering of *Chlamydomonas reinhardtii's* lipid metabolism is discussed. Using a strategy developed in higher plants, the lipid profile of *Chlamydomonas* was altered in order to increase the production of very long chain fatty acids. Finding alternate robust sources of the omega-3 very long chain fatty acids will be critical to alleviate the pressure placed on global fish reserves. Currently our primary source of omega-3 fatty acids comes from oceanic fish, which has led to dramatically dwindling populations due to overfishing. Since microalgae such as diatoms are the ultimate source of omega-3 fatty acids in fish, it is logical to use them as our production source of omega-3 fats. Additionally, the ability to manipulate lipid metabolism is the first step to addressing not only food shortages, but also in developing strains of microalgae for fuel production. Although fuels would typically use shorter chain fatty acids as their ideal inputs, work that shows the ability to modify lipid profile and helps scientists understand the impacts of changes to lipid profile will advance the field as a whole.

Chapter 4 discusses the microalgae *Chlorella,* a genus which contains several noteworthy species. *Chlorella* are well known for their ability to grow to very high density and their exceptional overall productivity. They are widely regarded as potential production strains of microalgae, although transformation of *Chlorella* has been difficult. Many transformation techniques were attempted and new constructs developed which may prove useful to other researchers trying to develop reliable nuclear transformation of *Chlorella*. Additionally, the growth rate and productivity of a variety of species of *Chlorella* was determined and the genome of one species sequenced. Genomic sequencing and assembly has enabled production of new transformation constructs and will enable new insights into the evolutionary history of this remarkable genus. Despite technical challenges and intractability, *Chlorella* remains a microalgae of great interest for the algae community and for industrial production.

The research described here provides a significant step forward in the field of algal lipid metabolic engineering, cultivation, and understanding of production strains. The ability to tailor lipid profile to suit the needs of industry will be critical for the advancement of microalgae as a viable prospect for both food and fuel. Rapid screening and crop assessment techniques will be required to enhance the profitability and reduce the costs associated with algae cultivation and help build a thriving new sector of the economy. Taken together, this work serves to help bolster the strength of the growing algae cultivation industry and to help it branch out into multiple markets.

CHAPTER 1:

MODIFICATIONS OF THE METABOLIC PATHWAYS OF LIPID AND TRIACYLGLYCEROL PRODUCTION IN MICROALGAE

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MICROBIAL CELL FACTORIES

REVIEW Open Access

Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae

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Abstract

Microalgae have presented themselves as a strong candidate to replace diminishing oil reserves as a source of lipids for biofuels. Here we describe successful modifications of terrestrial plant lipid content which increase overall lipid production or shift the balance of lipid production towards lipid varieties more useful for biofuel production. Our discussion ranges from the biosynthetic pathways and rate limiting steps of triacylglycerol formation to enzymes required for the formation of triacylglycerol containing exotic lipids. Secondarily, we discuss techniques for genetic engineering and modification of various microalgae which can be combined with insights gained from research in higher plants to aid in the creation of production strains of microalgae.

Introduction

In the past decade, the price of crude oil has ranged from 20 dollars a barrel to nearly 170 dollars a barrel. The volatile price, expected depletion and increase in atmospheric greenhouse gases due to oil combustion provide impetus to develop alternative energy sources. Biofuels have served as sources of energy from the beginning of human history, but the start of the industrial revolution led to a reliance on fossil energy due to its prevalence and high energy yields compared to the majority of bioenergy [1]. Establishing energy independence in coordination with the increasing costs for liquid fuels have renewed interest by the government, industry and academia in renewable liquid fuels to replace petroleum.

Biofuels can be solids, liquids or gasses so long as they are derived directly from biological sources. The most common solid biofuel is lignified cellulose (wood) that can be burned for energy. Liquid and gaseous biofuels generally require more refining, and include bioethanol, biodiesel, and engine-combustible hydrocarbons as well as methane from anaerobic digestion. The aforementioned liquid biofuels offer significant potential to augment or replace petroleum gasoline for transportation purposes. Currently ethanol dominates the biofuel market and may be produced by a variety of methods, primarily heterotrophic fermentation of sugars purified from biomass feedstocks [2]. Biodiesel, and other hydrotreated biofuels, are derived mainly from vegetable oil feedstocks (lipids) [3].

The lipids used for biofuels have important physiological roles in plants, including energy storage, structural support as membranes, and intercellular signaling [4]. Storage lipids differ from both structural and signaling lipids in that they are mainly composed of glycerol esters of fatty acids, also known as triacylglycerol (TAG). These lipids are generally stored in a compartment specialized for lipid storage, the lipid body. This compartment is found in most oleaginous plant cells, and is used to store a variety of TAG molecules depending on the species [5]. Vascular plants store large amounts of lipids in seeds, and provide energy for growth during germination. The lipid content, and fatty acid composition of oilseeds varies. Environmental changes or human manipulation, such as breeding or genetic engineering have been used to change lipid content and composition [6]. Although less common, some species like *Simmondsia chinensis* accumulate storage lipids as waxes rather than as TAG. Regardless of the final storage type, *de novo* fatty acid biosynthesis in plants occurs exclusively in the stroma of plastids, whereas, with the exception of plastidial desaturation and a few complex lipid biosyntheses, most modifications of fatty acyl residues and TAG synthesis from acyl chains are localized in the lumen of the endoplasmic reticulum (ER) [6]. In addition to TAGs, plants also contain membrane lipids. These,

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Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

unlike TAGs, remain highly conserved in both identity and quantity to maintain normal plant physiology.

Ethanol and biodiesel is primarily derived from plant sources, often food crops, because the established scale of food crops made them a convenient source of biomass necessary to produce biofuel on a commercial scale. However, an increasing demand for biofuel feedstocks has negatively impacted food markets, and raised a global "food vs. fuel" controversy. Furthermore, the land and fresh water requirements for growing crops, and the long growth-to-harvest periods limit the expansion of plant based biofuel industries to the amount of arable land. In contrast, unicellular algae require smaller amounts of land that does not need to be arable, have faster growing cycles, contain a higher percentage of oil, and have been proposed to be a better solution to the food vs. fuel debate. Therefore, significant attention has been focused on algae as a next generation feedstock for biofuel production [7]. It has been proposed that a fuel only based approach to biodiesel production from algae is unlikely to be feasible with current yields based on economic modeling of production facilities. As a result, attention must be paid to genetic manipulations in order to harness the ability of algae to make high quality fuel, but also potentially to serve as a factory for the production of other value added products such as protein therapeutics [8,9]. In light of this and studies on selection pressure for photosynthetic efficiency in native vs. bioreactor environments, it seems genetic modification is likely to provide the key to unlocking the feasibility of algal production strains [10].

Several research papers and reviews have been published presenting the recent progress in plant lipid biosynthesis and related industrial applications [4,11-14]. In this review, we discuss lipid biosynthesis and regulation in plants and algae; the state of genetic manipulation in plants to modify lipid biosynthesis; and the possible impacts of manipulation on biodiesel production from algae and future studies.

Biosynthesis of Triacylglycerol (TAG) in Plants

A general scheme of plant TAG biosynthesis is broadly discussed in the textbook "Plant Lipid Biosynthesis: Fundamentals and Agricultural Application" and other review articles, as shown in Figure 1. The TAG pathway begins with the basic fatty acid precursor, acetyl-CoA, and continues through fatty acid biosynthesis, complex lipid assembly, and saturated fatty acid modification, until finally reaching TAG formation and storage [15].

The fatty acid synthase (FAS) complex residing in plant chroloplasts is a major player in *de novo* fatty acid synthesis [16]. Completion of *de novo* fatty acid synthesis is accomplished in one of three ways [4,6]. Either the newly synthesized fatty acid is hydrolysed by a thioesterase, further modified by desaturases, or directly transferred to complex lipid formation ('prokaryotic lipid') using plastid acyltransferases. After release from plastids, free fatty acids are exported to the cytosol by an unknown mechanism and converted to acyl-CoA esters by an acyl-CoA synthetase located in the outer envelope of the plastid [17,18]. The cytosolic acyl-CoA esters are then transferred to the ER for further elongation, modification, or participation in the synthesis of membrane lipids or storage TAGs ('eukaryotic lipid').

Triacylglycerols (TAGs) are commonly found as storage fats or oils and are described as neutral or non-polar lipids, differentiating them from polar membrane lipids. TAGs consist of three FA chains esterified via the hydroxyl groups of a glycerol backbone. Biosynthesis of TAGs occurs in the plastids, mitochondria, and endomembrane system. Although the substrates are commutable, each compartment of the plant cell has an independent TAG synthesis pathway. The Kennedy pathway is well understood and one of the most straightforward TAG biosynthesis pathways; it consists of stepwise acylation, adding to each hydroxyl group of glycerol beginning with glycerol-3-phosphate [19]. Lipid bodies are single-layer, membrane-wrapped, protein embedded organelles 0.2-2.5 μm in diameter and are in the cytoplasm of most, if not all, plant cells [4,5]. It is generally believed that plant lipid bodies are not only a cellular lipid reservoir, but also provide an effective energy battery during seed germination. Plastoglobules are lipid bodies found within plastids that contain TAG, isoprenoid-derived metabolites, as well as proteins [20].

Palmitate (16:0) and stearate (18:0) are the major products of plastid FAS. However, the major fatty acids of plants are the C18 compounds, oleate (18:1Δ9), linoleate (18:2Δ9, 12) and α-linolenate (18:3Δ9, 12, 15). Together, these three fatty acids represent over 85% of total membrane acids, and over 80% of economically important storage oils. There are other various fatty acids which contain longer carbon-chains, unsaturated double bonds, hydroxyl groups, and other modifications within the plant fatty acid repertoire [21].

Fatty acid modification during plant lipid biosynthesis is crucial for generating the fatty acid repertoire found in plants. Fatty acids with 20 or more carbon atoms are called very-long-chain fatty acids (VLCFAs). In plants, VLCFAs are ubiquitous in leaf surfaces as wax, and in cuticle components which play an important role against xenobiotics. In *Simmondsia chinensis* seed, VLCFA is the major component of energy storage in the form of liquid wax consisting of chains 36 to 46 carbon atoms in length [11,13]. The VLCFAs are precursors of very-long-chain polyunsaturated fatty acids (VLC-PUFAs) which are important in human nutrition and health [22]. However, none of these VLC-PUFAs is normally produced in

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

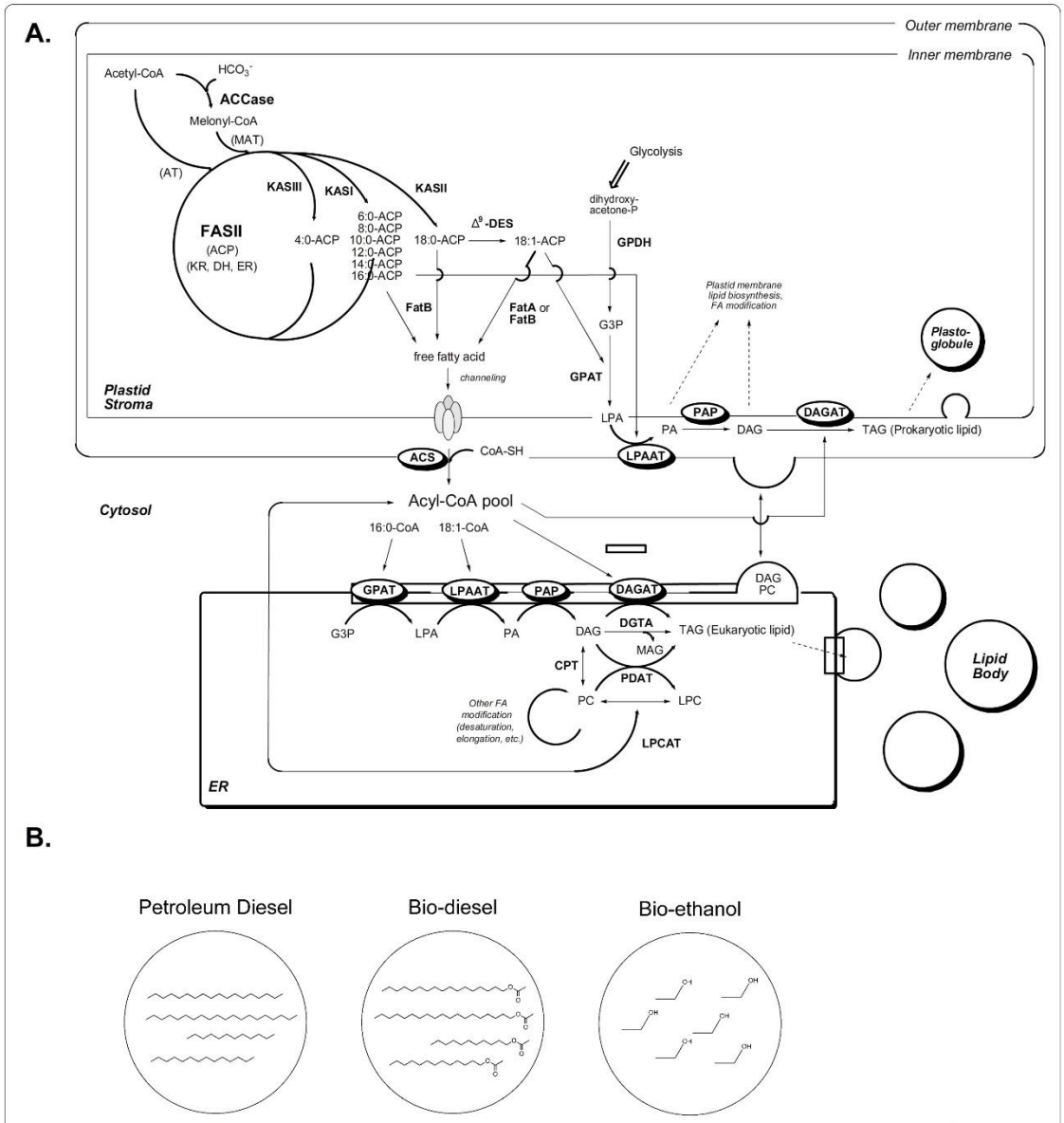

Figure 1 The general scheme of plant lipid biosynthetic pathway and representative chemical structures of petroleum diesel, biodiesel, and ethanol. AT = acetyltransferase, MAT = malonyl-CoA acetyltransferase, ACP = acyl carrier protein, KAS = ketoacyl synthase, FAS = fatty acid biosynthesis, KR = ketoreductase, DH = dehydratase, ER = enoyl reductase, GPDH = glycerol-3-phosphate dehydrogenase, GPAT = glycerol-3-phosphate acyltransferase, LPAAT = lysophosphatidic acid acyltransferase, PAP = phosphatidic acid phosphatase, DAGAT = diacylglycerol acyltransferase, ACS = acetyl-CoA synthetase, DGTA = diacylglyceryl hydroxymethyltrimethyl-β-alanine, CPT = carnitine palmitoyl transferase, PDAT = phospholipid diacylglycerol acyltransferase, LPCAT = lysophosphatidylcholine acyltransferase.

higher plants. In lower eukaryotes such as mosses, fungi, and algae, VLC-PUFAs are synthesized to confer flexibility, fluidity, and selective permeability to cellular membranes in stringent environments [23].

Biosynthesis of Triacylglycerol (TAG) in Algae

Algae are a diverse group of organisms which includes prokaryotes and eukaryotes in the form of single cells, colonized cells, and multicellular plants. Algae are

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typically distinguished from other classes of organisms by their ability to fix carbon, and utilize solar energy. Algae reside in a variety of ecosystems including marine and freshwater environments, desert sands, hot springs, and even snow and ice. To survive in these environments, algae produce a myriad of lipids. These include structural lipids for cellular membranes, as well as lipids for nutrient storage [24,25]. The two oil crises during the 1970's spurred a vigorous search for alternative energy sources, as people began to address their growing energy problem. From 1978 to 1996, the U.S. Department of Energy's Office of Fuels Development developed the Aquatic Species Program (ASP) with a goal of developing renewable transportation fuels from algae. During this program, systematic and fuel-directed algal oil research evaluated the potential of algal oil as an energy source. Although the program was terminated in 1996, the preliminary results of the pioneering studies provide a direction for later exploration in this field [26].

Over almost two decades of the ASP program, thousands of algae strains were isolated and screened for their lipid and fatty acid content. These data were combined with previous sporadic results, and some generalizations of lipid content in different algae categories were formed [26-29]. For example, diatoms are among the most common and widely distributed groups of algae. They store energy primarily in the form of lipids (TAGs) and the average lipid content of oleaginous diatoms is 22.7% dry-cell-weight (DCW) under normal growth conditions; with that number rising to 44.6% DCW when cultured under stress conditions [30]. However, the slower growth rate caused by nutrient deficiency, along with the increasing cost of silicate containing culture media hampers the usage of diatoms as a robust biofuel feedstock.

Green algae, often referred to as chlorophytes, are highly abundant and are estimated to number as many as 8,000 species. They are the most diverse group of algae, and include unicellular, colonial, coccoid, filamentous, and multicellular forms growing in a variety of habitats. Green algae are believed to share a common ancestor with higher plants, carrying the same photosynthetic pigments and having similar metabolic mechanisms. Generally, these algae use starch as their primary storage vehicle, however, in some strains large quantities of TAG accumulate under specific growing conditions. Oleaginous green algae contain an average total lipid content of 25.5% DCW, which can be raised to 55.2% DCW when the algae are grown under stress conditions or heterotrophically [30,31]. *Chlamydomonas reinhardtii* has been treated as a model organism for photosynthesis, and as a result has been studied extensively, because of its giant chloroplast and ability to control sexual reproduction, allowing detailed genetic analysis [32]. Indeed, *Chlamydomonas* was also the first alga to be genetically transformed and a draft sequence of the whole genome has recently been determined [33]. Although it does not typically accumulate lipids under ideal conditions, metabolic engineering can be used to transform this alga into an oleaginous factory [34].

Algal lipid metabolism from *de novo* fatty acid biosynthesis to the formation of complex glycerolipids is similar to that of the plant cells. Higher plants have differentiated organs, each of which performs specific physiological functions, and contains specific biochemical pathways. Similarly to higher plants, algae process TAG into lipid droplets which are coated in a large number of proteins. Most of these are typical members of vesicular transport and signaling pathways such as RabGTPases, but a proteomics approach to algal lipid bodies has identified a protein called major lipid droplet protein (MLDP) which affecs the size of lipid droplets and may present a target for immunofluorescence imaging of algal lipid content [35]. Algae species, especially microalgae, have a general biochemical composition of 30-50% DCW proteins, 20-40% DCW carbohydrates and 8-15% DCW lipids under optimal growth condition[36]. Most of the algal lipids are glycerinated membrane lipids, with minor contributions to overall lipid content from TAG, wax esters, hydrocarbons, sterols, and prenyl derivatives [30,36]. Under unfavorable growing conditions many algae shift their metabolic pathways toward the biosynthesis of storage lipids or polysaccharides. TAG accumulation in response to environmental stress likely occurs as a means of providing an energy deposit that can be readily catabolized in response to a more favorable environment to allow rapid growth [27]. Nutrients, temperature, light, salinity and growing phase have been shown to influence the flux of algal cellular metabolism [37].

Since many of the algal lipid metabolism studies on environmental changes have been carried out in batch cultures, there is a lack of systematic, multi-factor monitored studies. This decreases the practicability of applying previous findings to large-scale algal cultures. During the years of Aquatic Species Program, a 'silver bullet' was sought; a single species which could produce high levels of storage lipids without growth rate alteration. To maximize lipid production and growth efficiency for industrial scale culture, experiments with recombinant genetics and complex culture conditions (multi-stage cultures, timed nutrient limitations) may be required.

Engineering of Lipid Biosynthesis for the Production of Biofuels

1. Advantages of Biodiesel

Petroleum diesel or petrodiesel is a mixture of saturated and aromatic hydrocarbons with 10-15 carbon atoms and is ignited in high-compression diesel engines. Most plant oils (TAGs) are too viscous to use in modern diesel engines, and eventually lead to engine failure caused by

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

incomplete combustion. Biodiesel is mono-alkyl (usually methyl) esters (fatty acid methyl ester, or FAME) made by the transesterification of TAGs from vegetable oils or animal fats, and has a similar viscosity to petrodiesel [38]. There are several advantages in addition to carbon neutrality when using biodiesel as a liquid fuel source. The cetane number, a measure of the delay between compression and ignition, can be higher for biodiesel than regular grade petrodiesel. This reflects the quality of the fuel and a higher number is associated with shorter delays in ignition, resulting in more complete combustion. Burning biodiesel produces less carbon monoxide, particulate matter, sulfur, and aromatic compounds than burning petrodiesel. Furthermore, it has a higher flashpoint, allowing safer handling and storage and greater lubricity for engines than other fuels. It is made from renewable biomass and is biodegradable and "friendlier" to the environment than crude petroleum when fuel leakages do occur. Currently only two major renewable liquid fuels are produced in large quantities, bio-ethanol and biodiesel. Biodiesel has 25% higher energy content per volume, and requires much less energy input in production than bio-ethanol, as no distillation step is necessary. Additionally, ethanol has been shown to corrode pipelines, likely shortening their lifetimes [39].

Despite the many advantages, and increasing market share of biodiesel, there are limitations hindering its complete replacement of petrodiesel [38]. Negative biodiesel characteristics include poor cold-temperature properties, namely the tendency to solidify or gel, which can lead to fuel starvation and engine failure. The presence of polyunsaturated fatty acids in biodiesel also makes it susceptible to oxidation by atmospheric oxygen or hydrolytic degradation by water, which decrease the stability of biodiesel during long-term storage. In addition, the emissions from biodiesel contain a higher concentration of nitrogen oxide (NO_x) than do petrodiesel emissions, limiting its usage in areas under strict air quality standards. One of biodiesel's biggest limitations is cost and supply. As mentioned above, the use of oil crops for biodiesel production has already increased the cost of these commodities, and raised the 'food vs. fuel' debate. Although the oil supply problem may be relieved by switching from food plant to non-food plant feedstocks such as algae, the higher production costs of algal oil along with the lack of successful industry examples to date further hinders industry-scale adoption of algae-derived biodiesel.

The four major sources of plant oil today are oil palm, soybean, rapeseed, and sunflower, which together account for approximately 79% of the world's total production. Within these oils, palmitate (16:0), stearate (18:0), oleate (18:1Δ9), linoleate (18:2Δ9, 12), and α-linolenate (18:3Δ9, 12, 15) are the five main fatty acid components [14]. Unusual fatty acids produced by specific plant species contain unique functional groups giving them selective usages in industry [4]. The fatty acid composition determines the physical and chemical properties of the oil and its economic value. Traditionally, simple methods like blending or partial hydrogenation were applied to produce oils for specific applications. As the accumulating knowledge of plant lipid biosynthesis has been coupled with the development of advanced genetic technologies, various metabolic engineering methods have been performed to modify the fatty acid and lipid composition of several oleaginous plants [40-42].

2. Increasing Oil Content

Increasing oil content could be a straight-forward method to lower the high cost of biodiesel production, and may be applicable through genetic manipulation of lipid biosynthetic pathways. Table 1 shows an outline of genetic manipulations that have been performed in higher plants and the resulting changes in fatty acid composition and content. It has been proposed that lipid biosynthesis may be controlled by the availability of fatty acids, and that the production of fatty acids is regulated by acetyl CoA carboxylase (ACCase) [43,44]. Increasing the activity of ACCase may push excess substrate, malonyl-CoA, into the lipid biosynthesis pathway. Substantially increasing plastidial ACCase activity may prove quite complex due to the multigene-encoded enzyme complex and its post-translational regulation [45]. A successful example has been achieved by expressing a cytosolic version of the enzyme targeted to the rapeseed chloroplast [46]. This manipulation resulted in a higher ACCase activity and consequently a 5% increase in seed oil content, a relatively modest increase.

Increasing malonyl-CoA substrate pools for de novo fatty acid biosynthesis resulted in only minor increases in seed oil yield. Fatty acid synthase has been suggested to be another rate-limiting regulator of lipid production and several studies have been performed where a single enzyme of the FAS complex is overexpressed. Heterologous overexpression of KAS III, the first condensing enzyme synthesizing 4C acyl chains, increased the proportion of palmitic acid (16:0) but decreased the total fatty acid content by 5-10% [47]. The accumulation of butyryl-ACP suggests that KAS I is the next rate-limiting enzyme. It seems unlikely that the up-regulation of any single enzyme will have a major positive effect on lipid biosynthetic flux. Multiple gene expression or activation of key regulators operating on the entire fatty acid biosynthetic pathway may have a more substantial effect on lipid production [48].

The second part of triacylglycerol biosynthesis is the Kennedy pathway, which depends on levels of glycerol-3-phosphate. Increasing the glycerol-3-phosphate levels in developing seeds by overexpression of a yeast gene encoding a cytosolic glycerol-3-phosphate dehydrogenase

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

Table 1 A list of genetic modifications to higher plants and their resulting changes in fatty acid content

Modification	Organism	Result	Reference
Expression of a cytosolic variant of endogenous ACCase	*Brassica napus*	5% increase in seed oil content	[40]
Expression of KASIII from *Spinacia oleracea*	*Brassica napus*	Increased palmitic acid proportion, decreased total fatty acids 5-10%	[41]
Saccharomyces cerevisiae G3p dehydrogenase (gpd1)expression	*Brassica napus*	40% increase in seed oil content	[43]
Carthamus tinctorius G3p acyltransferase (GPAT) expression	*Arabidopsis thaliana*	10-21% increase in seed oil content	[45]
Saccharomyces cerevisiae sn-2 acyltransferase (SLC1-1) expression	*Brassica napus*	53-121% increase in erucic acid content	[47]
Arabidopsis thaliana diacylglycerol acyltransferase (DGAT1) expression	*Brassica napus*	Increases in oil content and seed weight	[48]
Down regulation of FAD2 desaturase and FatB hydrolase	*Glycine max*	85% increase in oleic acid levels	[53-55]
Expression of *Coriandrum sativum* Δ4palmitoyl ACP desaturase	*Nicotiana tabacum*	< 10% of total fatty acid became palmitoleic acid	[56]
Expression of *Thunbergia alata* Δ6 ACP desaturase	*Arabidopsis thaliana*	< 10% of total fatty acid became palmitoleic acid	[57]
Expression of *Umbellularia californica* lauryl-ACP thioesterase	*Arabidopsis thaliana*	24% of total fatty acid converted to laurate	[66]
Expression of *Umbellularia californica* lauryl-ACP thioesterase	*Brassica napus*	58% of total fatty acid converted to laurate	[67]
Expression of *Cuphea hookeriana* FatB1 thioesterase	*Brassica napus*	Fatty acid content changed to 11% caprylate and 27% caprate	[68]
Co-expression of *Cuphea hookeriana* FatB1 thioesterase and KAS (ketoacyl ACP synthase)	*Brassica napus*	30-40% increase in short chain fatty acid content over FatB1 expression only	[69]
Co-expression of *Cuphea hookeriana* FatB1 thioesterase and LPAAT from *Cocos nucifera*	*Brassica napus*	67% of total fatty acid content converted to laurate	[70]

(*gpd1*) resulted in a substantial increase in seed oil content up to 40% in transgenic rape [49,50]. Other successful examples increasing plant oil levels have come by altering the acyltransferases of TAG biosynthesis. *Arabidopsis thaliana* has been transformed with a soluble safflower glycerol-3-phosphate acyltransferase (GPAT), where the plastidial targeting sequence was removed, and an *Escherichia coli* GPAT inserted. Seeds of both transgenic plants produced 10 to 21% more oil [51]. A yeast *sn*-2 acyltransferase gene (*SLC1-1*) was introduced into a high erucic acid (22:1Δ9)-containing *Brassica napus*. The resulting transgenic strain showed a substantial increase in seed oil content and an increase in the proportion of erucic acid [52]. The transgenic strain was later tested in the field, and exhibited a 53-121% increase in total erucic acid yield (weight/plot) [53]. Overexpression of the Arabidopsis *DGAT1* gene in the wild-type strain led to increased seed oil deposition and average seed weight [54]. A functional DGAT homologue, the *DGAT2* gene from the oleaginous fungus *Mortierella rammanniana* was overexpressed in soybean, and resulted in small but significant increases in seed oil content in both greenhouse and field tests [55].

Together, these studies indicate that increased metabolic flux towards oil production may be achieved by manipulations targeted at later steps in the TAG biosynthetic pathway. A reasonable explanation is that the consequences of activating early biosynthetic steps may be slowed by later rate-limiting steps, and excess intermediate products may be utilized by other metabolic pathways sharing the same intermediates of TAG biosynthesis. Metabolic modeling networks that simulate flux of fatty acids through TAG biosynthetic pathways should play an important part in developing strategies for future genetic manipulation. Actual values of the engineering results need to be properly calculated for whole organisms and total production costs, not just the oil itself. For example, increasing oil content of soybean usually comes at the expense of the reduction of high-value protein content used for animal feed. Rigorous field testing is necessary to determine whether oil content increases are reflected in an increased oil yield per hectare per year. These tests must prove that strains with lipid content increases are economically viable compared to elite, high-yield commercial varieties.

3. Changing the Fatty Acid Composition of Oil

Beyond base supply, biodiesel has other limitations hindering its market competitiveness. The fuel properties of biodiesel are closely related to its fatty acid composition. Altering the fatty acid profile, for example the carbon chain length and number of double bonds, can lead to a better-quality, inexpensive biodiesel. The presence of methyl ester with saturated acyl chain longer than C12 significantly increases the cloud point of the biodiesel, the temperature at which crystals form [56]. The methyl esters derived from poly-unsaturated fatty acids are prone to oxidation and the hydroperoxides formed will

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

eventually polymerize and form insoluble sediments capable of interfering with engine performance [57]. Highly saturated and longer carbon chain esters have lower NO_x emissions relative to shorter, less conjugated chains [58]. In addition, biodiesel ignition quality is adversely effected by an increase in the number of double bonds [38]. When requirements for biodiesel quality are viewed together, it is clear no single fatty acid methyl ester (FAME) could fulfill every parameter. However, a balance of different fatty acids containing higher amounts of mono-unsaturated fatty acids such as oleate (18:1Δ9), and fewer saturated and polyunsaturated fatty acids would yield a more reliable biodiesel [59].

Increasing the contents of monoenoic fatty acids Most polyunsaturated fatty acids in storage lipids are derived from oleic acid by the catalysis of FAD2 (ω6) homologues. Therefore, down-regulation of the ER membrane-bound fatty acid desaturases should result in an increased percentage of oleic acid present, relative to total fatty acid content. Several experiments have successfully enhanced the oleate concentration in various oleaginous plants [60-62]. Down-regulating *FAD2* and *FatB*, which hydrolyzes the saturated acyl-ACP, further increases oleic acid levels in transgenic soybean to over 85%, with saturated fatty acid levels at less than 6%. In addition to oleic acid, other unusual monoenoic fatty acids from plants have potential for biodiesel production. Introduction of a coriander Δ4 palmitoyl (16:0)-ACP desaturase, or a *Thunbergia* Δ6 palmitoyl-ACP desaturase into tobacco callus and *Arabidopsis* seed, respectively, resulted in less than a 10% accumulation of these non-native unusual fatty acids and their derivatives [63,64]. Similar experiments have been performed in *Arabidopsis* and *Brassica napus* where Δ9 palmitoyl-ACP desaturase from *Uncaria tomentosa* was introduced. Significant increases in palmitoleic acid (16:1Δ9) and its derivatives were found in both transgenic plants, although the proportion of palmitoleic acid to total fatty acid content was much lower than the original *Uncaria tomentosa* (80%) [65]. The reason for the low levels of unusual monoene production in non-native plants may be lack of corresponding ACP, ferredoxin, 3-ketoacyl-ACP synthase, thioesterase, and acyltransferase present in the original strains [64,66]. Since fatty acid desaturases are highly conserved in their structure and amino acid sequences, several chimeric enzymes have been generated and shown to have broader substrate specificity [67,68]. These engineered desaturases may be more effective when designing transgenic plants to produce large amounts of monoenoic fatty acids [69].

Engineering of fatty acid chain length As mentioned previously, fatty acyl chain length is another important factor that influences the viscosity and cold flow properties of biodiesel [38]. Short- to medium-chain fatty acids (C8-C14) have lower viscosity and higher cloud points than common long-chain fatty acids (C16-C18). Although cold-flow properties are superior, cetane numbers are lower, and overall NO_x emissions higher for shorter chain fatty acids. However, increasing their proportion in market-available biodiesel still leads to better quality, more competitive fuel in terms of combustion performance.

Commercial oils from palm kernel and coconut oil contain > 40% of total fatty acids in the form of lauric acid (12:0). Plants that accumulate short- to medium-chain (C8 to C14) fatty acids in seed oil contain chain-length-specific acyl-ACP thioesterases that cleave the corresponding fatty acids from the growing acyl-ACP of *de novo* fatty acid biosynthesis [70]. For example, *Umbellularia californica* and *Cuphea hookeriana* seeds accumulate up to 90% short- and medium-chain saturated fatty acids in triacylglycerols. The chain-length-specific acyl-ACP thioesterases were identified in both species as the cause of the unusual accumulation [71,72]. The expression of a lauryl-ACP thioesterase from *Umbellularia californica* in the seeds of non-laurate-accumulating plants, *Arabidopsis* and *Brassica napus* (rapeseed), resulted in laurate quantities as large as 24 and 58% of total seed fatty acids, respectively [73,74]. In another transgenic experiment, a medium-chain thioesterase, Ch FatB1 from *Cuphea hookeriana*, which produces 50% caprylate (8:0) and 25% caprate (10:0) in their total fatty acids, was introduced into rapeseed. The transgenic rapeseed was found to accumulate up to 11% caprylate, and 27% caprate [75]. The reasons for lower production of short-chain fatty acids in transgenic hosts compared to donor species were further investigated. A short-chain-fatty-acid-specific condensing enzyme (3-ketoacyl-ACP synthase, KAS) from *Cuphea hookeriana* was identified and co-expressed with Ch FatB1 in rapeseeds. All double-transgenic lines showed a 30-40% increase in the levels of short-chain fatty acids compared to the Ch FatB1 single-transgene rapeseeds [76]. Additionally, structural analysis of TAG from the plants containing inserted medium-chain acyl-ACP thioesterase revealed that laurate was only present at *sn*-1 and *sn*-3 positions [74]. The high specificity of lysophosphatidic acid acyltransferase (LPAAT) from the hosts prevented laurate from being incorporated at the *sn*-2 position of TAG. Co-expression of a laurate-specific coconut LPAAT into rapeseed containing the *Umbellularia californica* thioesterase resulted in further increases in laurate levels, up to 67% of the total fatty acid content [77]. Another lesson learned from the study of laurate-producing transgenic plants was the importance of enzymes for lauryl-CoA β-oxidation, malate dehydrogenase, and isocitrate lyase, all of which participate in the glyoxylate cycle for fatty acid carbon reutilization. These genes were induced with increasing levels of the lauric acid [78]. Obtaining significant amounts of short-chain fatty acids in TAG may require the engineering of multiple

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

genes, including the short-chain-specific keto-synthase and thioesterase, as well as short-chain-specific acyltransferases, which assemble the novel fatty acids into TAG. Production of unusual fatty acids in transgenic hosts can induce antagonistic pathways reducing the effects of genetic manipulation, which must be addressed to maximize production efficiency.

Recently, direct use of low-molecular-weight TAG as fuel has been discussed and studied [59,79]. The lower cost of TAG fuels on the transesterfication and purification of FAMEs greatly enhances the market potential of such biodiesels. Seed oil containing 40% of caprylate (8:0) and 37% caprate (10:0) in total fatty acids from a mutant *Cuphea viscosissimal* had a coking index (a measure of engine carbon deposition) comparable to that of No. 2 diesel used by on road vehicles in the US, albeit with the problem of poor low-temperature viscosity [80,81]. Another interesting study involves the 1,2-diacyl-3-acetyl-*sn*-glycerols (ac-TAG) from the seeds of *Euonymus alatus* (Burning Bush). This acetyl TAG has a lower viscosity than common TAGs, and the potential to be used directly as biodiesel [59]. This specific acetyl DAGAT has been isolated from *Euonymus alatus*, and data on the oil properties of transgenic plants are much anticipated [82].

4. Manipulation of Algal Lipid Metabolism Using Genetic Engineering

During the years of ASP (Aquatic Species Program), an extra-copy of the monomeric ACCase gene was introduced into the genome of the diatom *Cyclotella cryptica*, in an attempt to increase lipid accumulation in the transformed strains [83]. Unfortunately, a two to three-fold higher ACCase activity in the transformed algae did not result in any enhancement of lipid production [26]. A major reason very few positive engineering results have been achieved in algae lipid metabolism is the lack of a reliable nuclear transformation system like that used in higher plants. A more promising method of genetic engineering has been successfully established in the chloroplast of *Chlamydomonas reinhardtii* [84]. However, as the examples in vascular plants have shown, most of the critical enzymes controlling lipid biosynthesis and fatty acid modification reside in the cytoplasm.

Several transformation techniques have been developed to genetically engineer *C. reinhardtii* to express recombinant proteins from both the chloroplast and nuclear genomes. General transformation protocols such as electroporation, particle bombardment, silicon carbide whisker agitation, and even *Agrobacterium tumefaciens* have been shown to transform a number of diverse microalgae including both green and red algae, diatoms, and dinoflagellates [85-89]. Expression levels vary greatly depending on a number of factors including auto-attenuation of exogenous sequences, codon usage bias, GC content, and proteasome mediated degradation [90]. Improvements in nuclear expression of transgenes have been reported with combined codon usage optimization, endogenous 5'/3' UTRs, and inserting introns from endogenous genes [91,92]. Transformation and expression research in *C. reinhardtii* will likely translate to a better understanding of microalgae gene silencing mechanisms and therefore more effective means to prevent transgene silencing in a variety of microalgae species.

Transformation of the nuclear genome allows for inducible gene expression, targeting to subcellular compartments, and protein secretion [93]. Insertion typically occurs via non-homologous recombination, though homologous recombination is known to occur at a very low frequency [94]. Optimizing homologous recombination conditions should allow for the directed knockout of enzymes diverting carbon usage away from lipid production, or for the directed replacement of lipid synthesizing enzymes with more effective isozymes. High levels of transgene expression can be selected for by using antibiotic resistance genes in combination with transgenic constructs. Addition of the *ble* gene to a transgenic construct confers resistance to phleomycin and zeocin in a 1:1 drug:protein ratio and can be used to select for transformants with high expression levels [95].

Although the nuclear genome does not yet robustly support protein production on a scale viable for harvesting protein therapeutics such as antibodies, expression of cytosolic enzymes or signaling proteins which promote the production of storage lipids may reach high enough activity levels to significantly alter the overall lipid profile of the host microalgae. *C. reinhardtii* and the model organism diatom *Phaeodactylum tricornutum* are known to produce fatty acids under nitrogen starved conditions and deletion of sta6 (involved in starch biosynthesis) in *C. reinhardtii* significantly increased lipid production in response to nitrogen starvation [34,96]. Endogenous micro RNAs (miRNA) and RNAi machinery have been shown to function and knock down gene expression in *C. reinhardtii*, and furthermore selectable constructs for artificially knocking down gene expression using RNAi machinery have been developed, enabling a reverse genetics approach to probing gene function [97-99]. High-throughput screening by insertional mutagenesis could be followed up with an RNAi based approach to investigate pathways for regulators of stress response, which may yield a genetic mechanism to increase lipid yield while minimizing growth arrest in large scale cultures. RNAi of protein members of pathways involved in lipid catabolism such as lipase and proteins of the beta oxidation, glyoxylate, and gluconeogenesis may represent important modifications which could increase overall TAG content [100].

To date, there have been over 30 complete genome sequences of algae determined, with still more unpublished

Yu *et al. Microbial Cell Factories* 2011, **10**:91
http://www.microbialcellfactories.com/content/10/1/91

[30,101]. With this primary sequence data and the functional characterization of homologous plant genes in hand, we can more precisely determine the key regulators of algal lipid biosynthesis *in silico*. Work has already started in this field, including the first gene-expression profile of *C. reinhardtii* under hydrogen-producing conditions, which was recently reported [102]. RNA-seq analysis of *C. reinhardtii* under nitrogen depleted conditions revealed statistically significant increases in several lipid biosynthesis genes including KASI, FAT1, and DGAT and decreases in beta oxidation genes such as LCS. Many of these genes were expected to be upregulated in lipid producing conditions, but more thorough bioinformatic analysis should yield new targets for genetic manipulation. More genomic, proteomic and metabolomic studies on algae lipid biosynthesis should also be nearing completion. The idea of algal oils as a potential biodiesel feedstock has been proposed and developed for years. The progresses in algal genetic engineering technology should accelerate any steps taken in achieving this goal.

Concluding Remarks

Renewable energy has become an important issue of recent political campaigns, and an increase in usage with less reliance on fossil energy will create substantial benefits for the global environment, economy, and industry. Biofuels are one of the few renewable energies proposed that have generated large public expectation as a real possibility for one of the fuels of the future. The use and production of plant oil as a source of biodiesel is expanding annually. Decades of studies have provided a general scheme of the plant lipid metabolism, and genetic engineering methods have provided valuable data and several field trials. However, more studies in organism-scale metabolic regulation will be necessary to understand how plants control their lipid biosynthetic pathways in response to physiological and environmental conditions. Elucidation of complex flux-control will hold great benefits for future biofuel production.

Algae, the world's largest group of photosynthetic organisms, contribute a majority of the carbon fixation on earth, turning greenhouse gases into carbohydrates and lipids. Using algal oils as a biodiesel feedstock holds major advantages in comparison to plant oils. Algal cultures have long been studied, and already are used to produce several important value-added products for the agriculture and food industries, such as VLC-PUFA, carotenoids, and high-protein animal feeds. The carbohydrates and cellulosic cell wall of algae have the potential to be hydrolyzed and fermented into bioethanol, further increasing the utility of algae as a biofuel feedstock. Algae cells can also be used to synthesize important eukaryotic proteins or natural products for pharmaceutical applications. Further fundamental studies in algae metabolism hold the possibility of making the algae cell a multi-use feedstock and creating a true "green gold".

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CHAPTER 2:

RAPID ESTIMATION OF PROTEIN, LIPID, AND DRY WEIGHT IN MICROALGAE USING A PORTABLE LED FLUOROMETER

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Short communication

Rapid estimation of protein, lipid, and dry weight in microalgae using a portable LED fluorometer

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ABSTRACT

As the industry surrounding microalgae continues to develop, there is a growing need for reliable, ready-to-use technologies for measuring the growth and composition of algal cultures. These can be new technologies or adapted existing technologies presently used for similar applications in other systems. Here we demonstrate the use of an LED fluorometer for the rapid estimation of dry weight, protein, and lipid content from two strains of microalgae. The instrument was successfully used to determine the dry weight of *Chlamydomonas reinhardtii* (CC-3491) and *Scenedesmus dimorphus* (UTEX 1237) cultures, at densities up to 1.58 g/L. Soluble protein was also measured using the instrument, and was highly comparable (average within 3%) to results obtained using both the Bradford and Lowry methods. Lastly, neutral lipid accumulation induced by nitrogen starvation was estimated via BODIPY 495/505 fluorescence. The basic methods developed here can easily be applied to any strain of microalgae or cyanobacteria, and demonstrate reliable, cost-effective, single-instrument methods for the determination of several key parameters in the cultivation of photosynthetic microorganisms.

1. Introduction

Microalgae's potential as a bio-product crop rests on its areal productivity and an ability to produce commercially relevant products. Accurate determination of productivity and biomass composition is key for both research and industrial production. Established methods for the measurement of biomass, protein, and lipid content are reliable and accurate, but are involved processes not well suited to portability due to the complex and expensive equipment required [1–3]. However, several analytical techniques and the associated instrumentation have recently seen large improvements in both portability and cost with little sacrifice in accuracy [4–6]. In the emerging field of algae biotechnology, portability and cost are two highly desired characteristics of any technique or instrument. Low-cost options for culture monitoring will become increasingly important as industries develop around microalgae, especially those attempting to compete with inexpensive commodity markets such as fuel and animal feed. It is with this scenario in mind that we tested and developed protocols for biomass, protein, and lipid determination using an inexpensive and commercially available LED fluorometer.

The assays described were developed using the Qubit 2.0, a portable LED fluorometer equipped with two LEDs: blue with a maximum of ~ 470 nm, and red with a maximum of ~ 635 nm (filters 430–495 nm and 600–645 nm, respectively). Two emission filters from 510–580 nm and 665–725 nm are built into the instrument. These optics make the instrument well suited for measuring chlorophyll fluorescence in microalgae without any modification, enabling the use of chlorophyll *a* fluorescence as a corollary for other parameters such as dry weight or culture density. Additionally, the instrument is spectrally compatible with lipophilic BODIPY dyes, and hence well suited for estimation of total lipid content. BODIPY fluorophores have long been used with fluorometers for total lipid determination, and their potential use expands well beyond this purpose [7,8]. In addition to lipid, a protein assay has been optimized for protein concentrations of 12.5 μg/mL–5 mg/mL and can tolerate between 1–20 μL of sample. The assay utilizes a solvatochromatic dye that exhibits a large increase in fluorescence signal in the presence of a protein–detergent complex. Several typical issues often encountered in protein assays, such as interference by salts and cellular contents such as DNA, free amino acids, and various solvents, are well-tolerated, and have been used to determine purified algal protein concentrations previously [9].

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Microalgae continue to grow as a production platform for biofuels, therapeutic proteins, and high-value products such as omega-3 oils and vitamins [10,11]. The two strains tested here represent model laboratory and production strains. *Chlamydomonas reinhardtii* has long served as a genetic and metabolomics model alga, and a photosynthetic bio-factory for recombinant proteins [12,13]. Outside of the laboratory, *Scenedesmus dimorphus* has proven an immensely promising bio-products strain due to its high tolerance of both abiotic and biotic stresses [14]. *Scenedesmus* sp. have recently been genetically manipulated, adding to the likelihood that the species will become an important producer in the emerging industry [15]. The ability of the fluorometer-based assays to work with both these species of microalgae, along with their relative speed demonstrates the potential of these assays to be versatile tools in the field of microalgae research.

2. Materials and methods

2.1. Maintenance of stock cultures

C. reinhardtii (*Cr*) (CC-3491) was obtained from the Chlamydomonas Resource Center, University of Minnesota, USA. *S. dimoprhus* (*Sd*) (UTEX 1237) was obtained from the University of Texas at Austin Culture Collection, Texas, USA. Strains were maintained on 1.5% agar plates of Sueoka's High Salt Medium (HSM) [16]. Stocks were maintained in ambient conditions at 24 °C under 6500 K fluorescent lights at a constant irradiance of 60 μmol m^{-2} s^{-1}.

2.2. Algal fluorescence

Autofluorescence was measured using the blue excitation and far-red emission channels in the Qubit 2.0 raw mode (470 nm excitation, 665–720 nm emission). For all measurements, at least 300 μL of sample was used. Sample tubes were 0.5 mL, thin-wall, clear PCR tubes.

2.3. Dry weight determination

Dry weights (DW) were obtained using fritted glass filter assemblies (ChemGlass 1424 assembly) and 4.7 cm Whatman GF-B glass fiber filter discs. Filters were prewashed five times using 10–15 mL of DI water and dried overnight in a 105 °C oven. Filters were allowed to cool in a desiccator before weighing. Weights were taken on an analytical balance readable to 0.1 mg. Fluorescence/dry weight correlation curves (Fig. 1) were created for each strain by measuring a series of dilutions created from culture samples.

2.4. Soluble protein

Cr and *Sd* were grown in 1 L flasks containing 250 mL of TAP media [17] at a temperature of 24 °C under a constant irradiance of 100 μmol m^{-2} s^{-1}. During logarithmic phase, at a cell density of $1 \cdot 10^6$ cells/mL, 250 mL of culture were spun at a relative centrifugal force (RCF) of 2000 g. The pelleted cells were then resuspended in 15 mL of tris-buffered saline (pH 7.6) and placed on ice. After 10 min

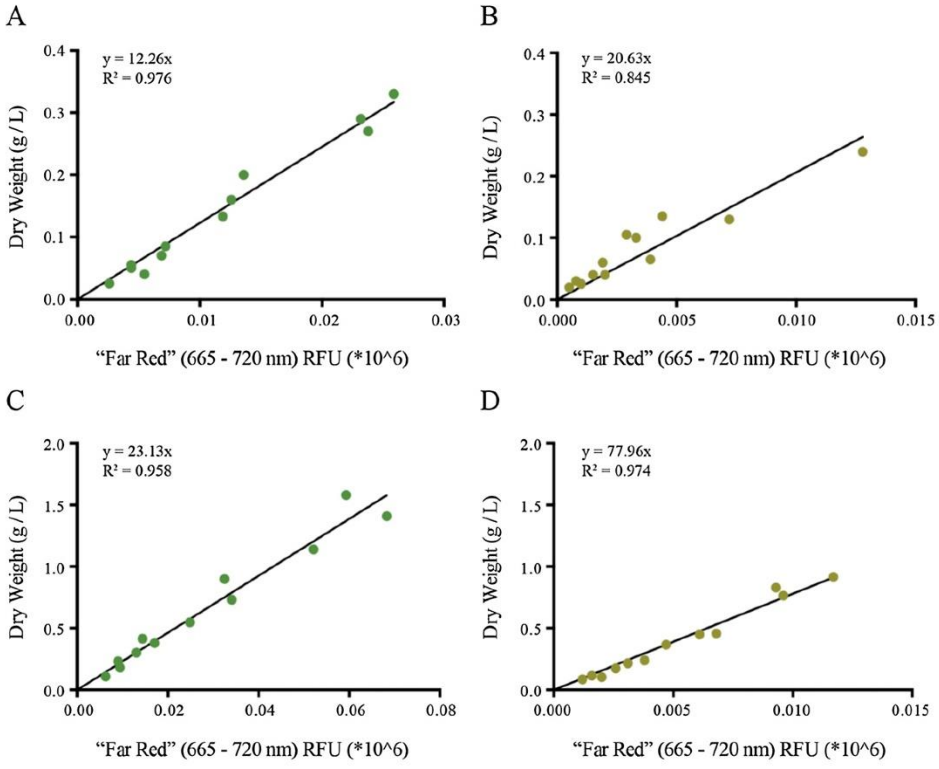

Fig. 1. Fluorescence–dry weight correlations for *Chlamydomonas reinhardtii* and *Scenedesmus dimorphus*; red (635 nm) excitation, far red (665–720 nm) emission. A) *Cr* nitrogen replete and B) nitrogen deplete culture fluorescence vs. dry weight. C) *Sd* nitrogen replete and D) nitrogen deplete culture fluorescence. Each plot shows sufficient linearity for dry weight determinations based on rapid fluorescence readings.

of cooling, cells were sonicated using a Fisher Scientific Sonic Dismembrator Model 500 at 15% amplitude in two 15 second bursts. Sonicated cells were then incubated on ice for 20 min and spun in 1.5 eppendorf tubes at 16,000 RCF in an Eppendorf tabletop centrifuge. Instructions for Bio-Rad Bradford and DC (Lowry) assays were followed for the microplate reader versions of the assay, while the Lifetech Qubit assay was performed using the Qubit 2.0 according to manufacturer's instructions. All assays were performed in three technical replicates.

2.5. Nitrogen starvation experiments

Growths for nitrogen starvation experiments were conducted using 50 mL cultures grown in a CO_2 chamber at 27.0 °C under 6500 K fluorescent lights at a constant irradiance of 95 μmol m^{-2} s^{-1}. Starter cultures were started from plates by scraping a portion of the plate and resuspending in 1 mL of the appropriate media before addition to the culture flask. Triplicate nitrogen replete and deplete cultures for nitrogen starvation experiments were inoculated from the same starter culture at a density of $1.0 \cdot 10^6$ and $5.0 \cdot 10^6$ cells/mL for *Cr* and *Sd* respectively. Cultures were grown for 72 h before being analyzed for lipid content. All cultures were grown in nitrogen replete or deplete tris-acetate-phosphate (TAP) media.

2.6. Lipid analysis

Following 72 h of growth, 1 mL samples from each culture were used for lipid estimation via BODIPY fluorescence. 5 μL of a 0.1 mM stock solution of BODIPY 495/505 in DMSO was added to each sample to yield a final dye concentration of 0.5 μM. After 30 min at room temperature cells were pelleted by centrifugation, washed twice with DI water, and resuspended in fresh media. The samples were then measured using the instrument's blue excitation (470 nm) and green emission filter (510–580 nm). A cell count was taken before and after washing. Samples of known concentration were also collected and prepared for GCMS analysis by resuspension in 0.5 mL of 1 M hydrochloric acid in methanol in Eppendorf tubes. Seven microliters of a 10 mM tridecanoic acid in methanol solution was added to each sample as an internal standard. The methanolic acid samples were incubated at 70 °C for one hour before being extracted twice with hexane. The hexane extracts of each sample were pooled in GCMS vials for analysis. The run was performed on 1 μL of sample with splitless injection. Samples were separated on a 60 m DB23 Agilent GCMS column using helium as carrier gas and a gradient of 110 °C to 200 °C at 15 °C/min, followed by 20 min at 200 °C.

3. Results

3.1. Algal fluorescence and dry weight determination

To determine total biomass density, a dry weight–autofluorescence curve was generated for both nitrogen replete and nitrogen deplete cultures of each experimental strain (Fig. 1). The two curves were necessary due to the expected variation in pigment concentration between the two conditions, likely as a result of cannibalization of photosynthetic protein–pigment complexes under nitrogen deplete conditions.

The linear range of algal fluorescence on the instrument was determined to be 0–$5 \cdot 10^6$ relative fluorescence units (RFU) using excitation/emission wavelengths of 470/665–720 nm, and 0–$8 \cdot 10^3$ RFU when using excitation/emission wavelengths of 635/665–720 nm. Measurements outside of the linear range were easily accomplished by sample dilution. Fluorescence vs. DW plots showed strong linearity for both strains tested (Fig. 1).

3.2. Soluble protein

Soluble protein measurements of each strain using both the Bradford and Lowry methods showed excellent agreement with concentrations measured with the fluorometer. Total soluble protein concentration of *Cr* was determined to be 2.17 ± 0.15, 2.10 ± 0.16, and 2.14 ± 0.06 μg/mL using the Bradford, Lowry, and Qubit assays respectively. Soluble protein level in *Sd* were determined to be 2.29 ± 0.07, 2.09 ± 0.18, 2.24 ± 0.01 μg/mL using the same assays. These results are averages and standard deviation from three technical replicates diluted to the linear range of each assay. Overall relative standard deviation between assays for both *Cr* and *Sd* was less than 5%.

3.3. Lipid content

Relative lipid content was determined using the fluorescence signal from BODIPY 495/505 stained samples. Measuring lipid via fluorescence provides a relative measure of lipid content, and a rapid comparison of populations of the same strain. For total lipid estimation, relative fluorescence can be correlated to total lipid [18], which is commonly measured via GC/MS. Under nitrogen deplete conditions, both strains showed an accumulation of lipid as measured by relative fluorescence at 72 h of growth (Fig. 2). This increase correlated to an increase in lipid levels as measured by FAME analysis via GC/MS.

Fluorescence signals were normalized to cell counts post-washing to account for losses during this step, and showed good correlation with lipid levels observed by GC/MS.

4. Discussion

4.1. Algal fluorescence and dry weight determination

The standard set of LEDs and emission filters on the instrument proved adequate for determination of chlorophyll fluorescence from the two strains of microalgae tested. In-depth studies on the use of chlorophyll autofluorescence as a means of estimating dry weight have been conducted [19], and here we present a basic correlation between the two for both nitrogen replete and deplete conditions. Although convenient and consistent for both strains tested, it is likely that chlorophyll fluorescence vs. dry weight correlation curves would need to be established for both indoor and outdoor conditions due to varying chlorophyll production under different light regimes. However, with the current speed of chlorophyll measurements using the instrument, dry weight measurements would remain the bottleneck. Typically, chlorophyll fluorescence is measured with an excitation wavelength of 440 nm, and emission wavelengths of 660–700 nm. The blue excitation LED (470 nm) coupled with the far-red emission filter (665–720 nm) proved adequate for chlorophyll fluorescence readings over a wide range of culture densities. Again, it is important to note that the slope of the best-fit line varies significantly between strains, and thus fluorescence vs. dry weight correlations must be obtained for each strain and growth condition under investigation. This is likely due to natural variations in pigment composition between various strains of microalgae [20]. Despite this limitation, both excitation wavelengths proved adequate for correlations, allowing for the possibility of dry weight and lipid determination with only two fluorescence readings on a single device.

4.2. Soluble protein

Fluorometers have been used previously in the determination of DNA concentration in microalgal cultures [21], and here we demonstrate their additional utility in measuring soluble protein concentration. Comparison of the DC and Bradford assays to the Lifetech fluorescent assay reveals consistent results between the three assays

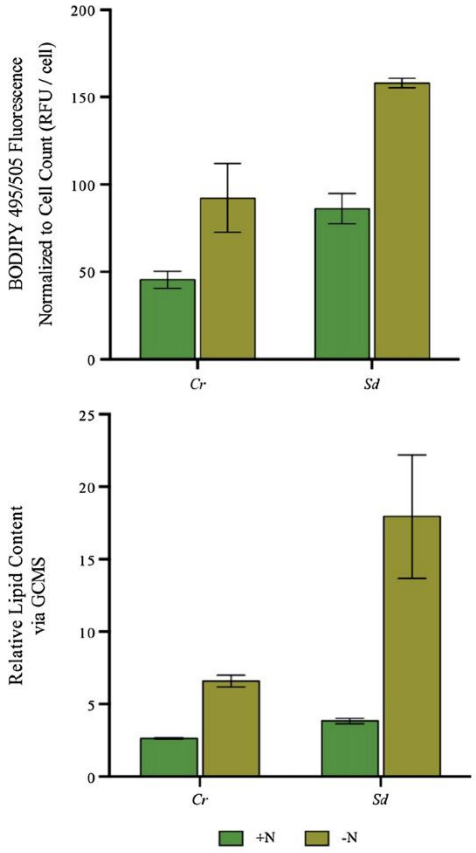

Fig. 2. Total lipid measured via BODIPY 495/505 fluorescence. Both *Cr* and *Sd* showed a marked increase in lipid, as expected, following 72 h of growth in nitrogen deplete media. Triplicate cultures were grown and tested for lipid content. For the speed of the assay, good correlation is shown between BODIPY fluorescence and GC/MS data.

despite their use of different measurement methods (absorbance vs. fluorescence) and wavelengths. Samples of algae lysate measured by all three techniques and between the two strains tested yielded values within 1–7% of each other.

Traditional assays for the measurement of protein content rely on the use of plate readers measuring absorbance from a protein binding dye. While these assays are widely used and reliable, they are not especially well suited to field measurements or transport of the instrumentation. The assay we present is capable of producing results with the same accuracy as traditional Bradford or Lowry protein assays using a portable fluorometer and fluorescent dye. The protein assay reagent interacts with detergent–protein complexes and when bound, fluoresces green in response to excitation at 420 nm. Additionally, Bradford and Lowry are affected by detergents and salts in solution and have a more narrow linear range at 0 to 2 mg/mL for Bradford and 0 to 0.25 mg/mL for Lowry compared to 0 to 5 mg/mL in the fluroscent protein assay. All three assays tested showed highly similar results, demonstrating the usefulness of LED fluorometry in measuring the total soluble protein content of microalgae cultures.

4.3. Lipid content

Total lipid is most accurately determined gravimetrically via solvent extraction, but this technique is not well suited to small-scale analysis. The process involves various drawbacks such as the need for large amounts of biomass, multiple solvent extractions, and relatively long processing times [3]. Faster measurements of lipid content require derivatization of triacylglycerols to fatty-acid esters followed by analysis using gas chromatography/mass spectrometry to provide a quantitative output. GC–MS instruments are typically expensive, although they are well suited to high-throughput, small-scale measurements. Variations can also exist in lipid content measured using GC–MS [22].

Here we have demonstrated the use of an LED fluorometer to obtain a rapid estimation of total lipid, which can then be correlated to traditional GC/MS or gravimetric measurements. The fluorescence signal of the dyes measured in the fluorometer provided a rapidly determined relative measure of lipid content (Fig. 2). This technique is best suited to determining differences in lipid content between two cultures of the same species of algae, due to the variation in baseline signal of different species. The difference in baseline signal in *S. dimorphus* (UTEX 1237) and *C. reinhardtii* (CC3491) is likely due to the difference in permeability of the Bodipy dyes between strains. *Cr* is a cell wall deficient mutant while *Sd* possesses a tough cell wall which could reduce permeability of the dye [23]. However, using this technique, qualitative comparisons between cultures of the two species showed good correlation to GC/MS measurements. This technique is well suited for obtaining lipid data during different growth phases which could rapidly be compared in order to optimize harvesting and growth processes based on lipid content. The techniques can be adapted to provide a quantitative assessment of lipid content by correlating the fluorescence data to a quantitative technique. Cell counts were a necessary part of the assay to account for strain variability and losses in the washing step, but this could be replaced using the previously discussed estimation of dry cell weight using chlorophyll by relating chlorophyll fluorescence to cell counts. Different species of algae have diverse cell wall types even within families of algae that could potentially lead to variation in BODIPY signal intensity for a given lipid content [24], but with proper controls, the fluorometer provides a rapid measure of total lipid. Lastly, while it may be difficult to compare total lipid levels between strains due to inherent differences in dye penetration, our results demonstrate that lipid accumulation in a single strain under different environmental conditions can be rapidly assayed using the fluorometer.

5. Conclusion

As techniques for the monitoring of growth and cell composition of microalgae expand, there will be a push for rapid assays and field instrumentation capable of producing accurate and reliable measurements. We have demonstrated that LED fluorometers can be used as quick and accurate instruments to assess biomass, soluble protein, and lipid content of microalgae cultures. Significant biodiversity of algae makes standardization of assays a challenge. However, the framework set in this study can serve as a foundation to apply these techniques to any strain of microalgae. The methods described here can be used for direct qualitative comparison between cultures of a strain, without the need for more time-consuming methods or expensive instrumentation.

Acknowledgments

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Acknowledgements

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CHAPTER 3:

METABOLIC ENGINEERING OF CHLAMYDOMONAS REINHARDTII TO PRODUCE EICOSADIENOIC AND EICOSATRIENOIC FATTY ACIDS THROUGH HETEROLOGOUS EXPRESSION OF A Δ9 ELONGASE

Abstract

A codon optimized gene encoding *IgASE1*, a Δ9 elongase from *Isochrysis galbana* with specificity for elongation of linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) was synthesized and cloned into a nuclear expression vector and transformed into *Chlamydomonas reinhardtii.* After PCR screening and subsequent small scale GC-MS testing, several transformants were identified, one of which produced eicosadienoic (C20:2n-6) and eicosatrienoic acid (C20:3n-3) at 4.2% and 1.2% of total fatty acids, respectively. Tandem TLC/GC-MS analysis of nitrogen deplete and refed cultures revealed that the elongase produced C20 fatty acids in the phosphatidylethanolamine and triacylglycerol lipid pools. Subsequent refeeding of nitrogen resulted in an increase in percentage of C20 lipids in TAG, indicating possible incompatibility with endogenous acyltransferases.

Introduction

The omega-3 long chain polyunsaturated fatty acids (LC-PUFAs) play an important role in the maintenance of human health and development, as they aid in brain and eye development during growth and are required for the differentiation of dopaminergic neurons during adulthood.[1, 2] Strategies by which their accumulation can be increased or engineered are an area of research interest since the majority of the world suffers from some form of essential fatty acid deficiency.[3]

Although omega-3 supplements are typically sourced from fish, microalgae are ultimately the source of the majority of omega-3 fatty acids found in our diets. Overfishing is a major obstacle in the growth of the omega-3 supply, which has expanded rapidly into a more than $10 billion per year market.[4] The next step in the growth of omega-3 supplements is to identify and engineer strains of algae that are extremely productive and capable of accumulating omega-3 fatty acids to high levels in order to alleviate the stress on global fish reserves.

Metabolic engineering has the potential to transform public health and alleviate the stress on fish stocks as a result of overfishing. Important advances have been made in engineering higher plants to produce omega-3 fatty acids but these techniques have not yet been applied to algae, which have a much greater potential yield per land use.[5] In *Brassica napus*, heterologous gene expression has been used in order to generate transgenic plants with eicosapentaenoic acid (EPA) at up to 26% of total leaf triacylglycerol (TAG).[6] This paper successfully demonstrates the first step in the metabolic engineering of microalgae to produce omega-3 long chain fatty acids and follow up studies will build upon these results in order to establish a means of generating an ideal algae strain for phototrophic production of omega-3s. Advancements in the field of metabolic engineering will allow not only the production of omega-3 fatty acids, but also enable custom tailoring of fatty acid profile in order to generate designer strains which can be used for the production of food or fuel products in the future.

Materials and Methods

Strains and cultivation conditions

The *Chlamydomonas reinhardtii* strain used in this work was CC1690, obtained from the *Chlamydomonas* Stock Center. Unless otherwise noted, algae were grown under constant irradiance at 150 μmol photons m^{-2} s^{-1} and shaken at 120 rpm. *C. reinhardtii* were grown in acetate enriched media using the standard TAP recipe described by Harris *et al*.[7] Nitrogen starvation media (TAP –N) was prepared according to Harris *et al.* (cited above) and cultures with $5x10^6$ cells mL^{-1} were spun at 600x RCF for 10 minutes at room temperature. Pellets were then washed twice with TAP –N and resuspended. For nitrogen refeeding experiments, the nitrogen starved cultures were spun at 600x RCF for 10 minutes at room temperature and pellets were washed twice with TAP media and then resuspended.

Construct design and transformation

The *Isochrysis galbana* Δ9 elongase gene (NCBI accession #ADD51571) was obtained from Life Technologies® and codon optimized to match the *C. reinhardtii* nuclear genome and cloned into expression vector pBR9. The gene was PCR amplified using the forward primer 5' CTC GAG ATG GCC ACC GAG GCC ACC 3' and reverse primer 5' GGA TCC CTA CAG GGC CTT GCC GCC 3' These primers incorporate Xho1 and BamH1 restriction sites which were used for cloning into the pBR9 vector. PCRs were performed in New England Biolabs® GC buffer with the following protocol: 98°C for 2 minutes, 98°C for 30 seconds, 63°C for 20 seconds, 72°C for 45 seconds, repeating all but the first step 35 times. Electroporation was performed as described previously by Rasala *et al.*.[8]

Fluorescence Microscopy

Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope with a Hamamatsu OrcaER C4742-95 charge-coupled-device camera and Simple PCI software version 6.1. Log phase cells were collected and 10μL deposited onto cooled .7% agar pads on a six well plate.

Western Blot

Western blots were performed as described by Cohen *et al.* 1998, using a rabbit anti-mCherry primary antibody (Abcam, San Francisco, CA, USA) and an alkaline phosphatase-conjugated goat antirabbit secondary antibody (Sigma, St Louis, MO, USA).[9]

Colony PCR

Pipette tips were used to obtain a small aliquot of each colony of algae grown on tris-acetate-phosphate (TAP) plates containing Zeocin at 10μg/mL. The tips were then placed in

separate wells of a PCR plate containing 15uL of 10X TE buffer and agitated to remove the algae from the pipette tip. Lysates were created by boiling the algae at 100°C for 10 minutes, after which the algae was spun in a centrifuge at 1000x RCF for 3 minutes. A 1μL sample of each colony lysate was used as a template for PCR under the same conditions as above.

GCMS and Lipid Analysis

Colonies were grown in TAP media under 150 μmol photons m^{-2} s^{-1} irradiance and prepared for GCMS analysis by spinning in a centrifuge at 2000x RCF. Pellets were resuspended in 0.5 mL of 0.5M sulfuric acid in methanol in glass test tubes. The methanolic acid samples were then incubated at 70 ˚C for one hour before extraction into 500uL hexane. The hexane was collected and 1μL of sample run with splitless injection using nonadecanoic acid (C19:0) as an internal standard. Samples were separated on an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C triple-axis mass detector using a 60m DB23 Agilent GCMS column using helium as carrier gas and a gradient of 110 ˚C to 200 ˚C at 15 ˚C/min, followed by 30 minutes at 200 ˚C.

TLC

Thin-layer chromatography was performed as described by Fan et. al. using the two stage process with development in acetone, toluene, and water (91:30:3, respectively) followed by hexane,diethyl ether, and acetic acid (70:30:1).[10] Fatty acid groups were visualized by copper sulfate staining and then scratched into separate vials for transesterification and GCMS analysis as described above.

RT-QPCR

RNA was prepared from log phase ($5x10^6$ cells mL^{-1}) cultures grown under 150 μmol photons m^{-2} s^{-1} irradiance using PureLink Plant RNA reagent using the manufacturer's protocol.

1µg of RNA was reverse transcribed using the Verso cDNA synthesis kit. cDNA was diluted 1:2 for RT-QPCR analysis using Power SYBR Green PCR Master Mix from Applied Biosystems. Data were analyzed according to the ΔΔCt method using *RACK1* as the reference gene. The figure shown plots transcript abundance normalized to the IG74 culture.

LCMS

50mL cultures were grown in TAP media under 150 µmol photons m^{-2} s^{-1} irradiance and prepared for LCMS analysis by spinning in a centrifuge at 2000x RCF. 100µL cell pellets were trypsin digested and iTRAQ labeled using instruments and according to the methods used by O'Brien *et al*.[11]

Results

Construct design and transformation

Figure 1A shows the aerobic pathway of omega-3 fatty acid production starting from *α*-linolenic acid and ending in the highly valued eicosapentaenoic acid (EPA, C20:5). The traditional and more commonly used pathway takes the left branch and occurs via Δ6 desaturation and subsequent Δ6 elongation. The less commonly found pathway shown in the right branch occurs in *Isochrysis galbana* via Δ9 elongation and includes a Δ8 desaturation.[12]

The *Isochrysis galbana* native Δ9 elongase gene *IgASE1* was codon optimized according to the nuclear codon bias of *Chlamydomonas reinhardtii.*[13] The elongase was then subcloned into the pBR9 nuclear transformation vector utilizing the Hsp70 promoter and the RbcS promoter to drive strong transcription (Fig 1B).[14] In order to select for high levels of protein accumulation, the elongase was placed downstream of the *Ble* gene coding for the Zeocin resistance protein. The Ble protein acts as a 1:1 inhibitor of the drug Zeocin because it acts by binding Zeocin directly rather than enzymatically through cleavage.[15] This allows selection of only the clones which accumulate

protein at high enough levels to ensure Zeocin inactivation, and may bias towards selection of insertions in highly transcribed regions of chromatin.

In order to take full advantage of the *Ble* gene, the 2A peptide linker is used between the *Ble* gene and the elongase in the construct. The 2A sequence is a special peptide from Foot and Mouth Disease Virus (FMDV) that allows for the production of two proteins from a single transcript by allowing the ribosome to skip a peptide bond during translation.[16] This allows *Ble* and *IgASE1* genes to be transcribed onto the same mRNA strand and links their expression directly.

Colonies that grew on TAP Zeocin transformation plates were patched onto numbered Zeocin plates and colony PCR was performed to detect the presence of the elongase gene (Fig 1C). Gene positive colonies were then scaled up to 8mL and grown in TAP media for GCMS analysis to detect elongase enzymatic function. After screening 500 gene positive transformants, eight colonies were found by GC-MS to produce both eicosadienoic acid (EDA, C20:2n-6) and eicosatrienoic acid (ETrA, C20:3n-3).

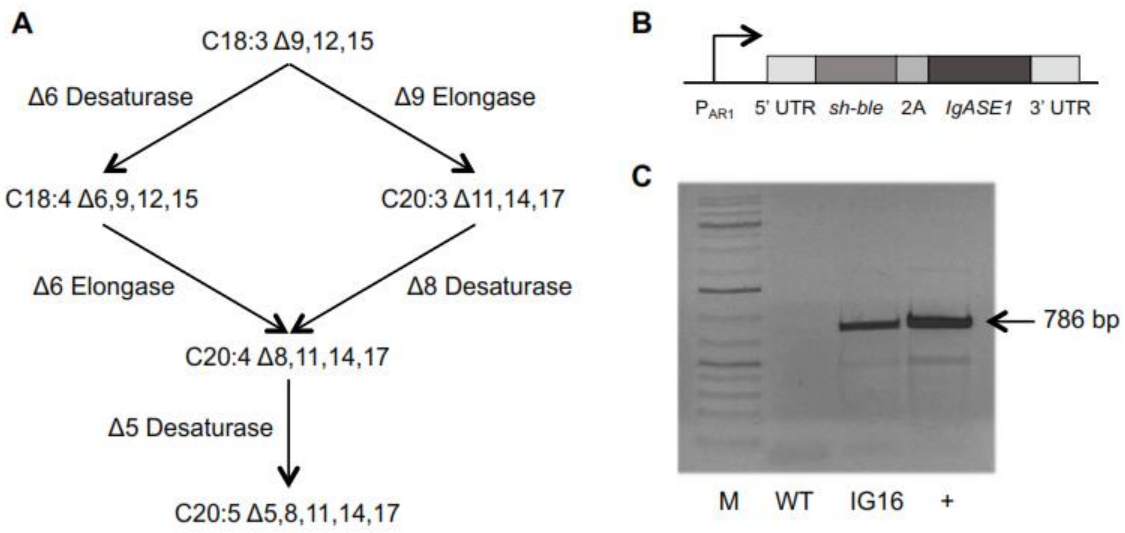

Figure 3.1 Construct Design and Transformation. A. Aerobic omega 3 pathway illustrating the Δ6 (left side) and Δ8 (right side) desaturase pathways to EPA. B. Construct diagram: P - RbcS/Hsp70 promoter, *sh-ble* - *Streptoalloteichus hindustanus* Zeocin resistance gene, 2A – Foot and mouth disease virus 2A sequence, *IgAse1* – *Isochrysis galbana* Δ9 elongase gene, 3'T – RbcS 3' UTR and terminator sequence C. Colony PCR showing 1kb plus ladder (lane M), wild type (lane 1), gene positive transformant (lane 2), and positive control amplified from vector (lane 3).

Constructs containing *IgASE1* fused to both FLAG and mCherry were generated to produce tags on both N and C terminal domains of the elongase protein. No functional elongase activity was detected in any microalgae transformed with these constructs. mCherry tagged constructs were examined by fluorescence microscopy and western blotting, which revealed that the mCherry tagged elongase was not present in the plastid and was present in the soluble protein fraction (Supp Fig 1).

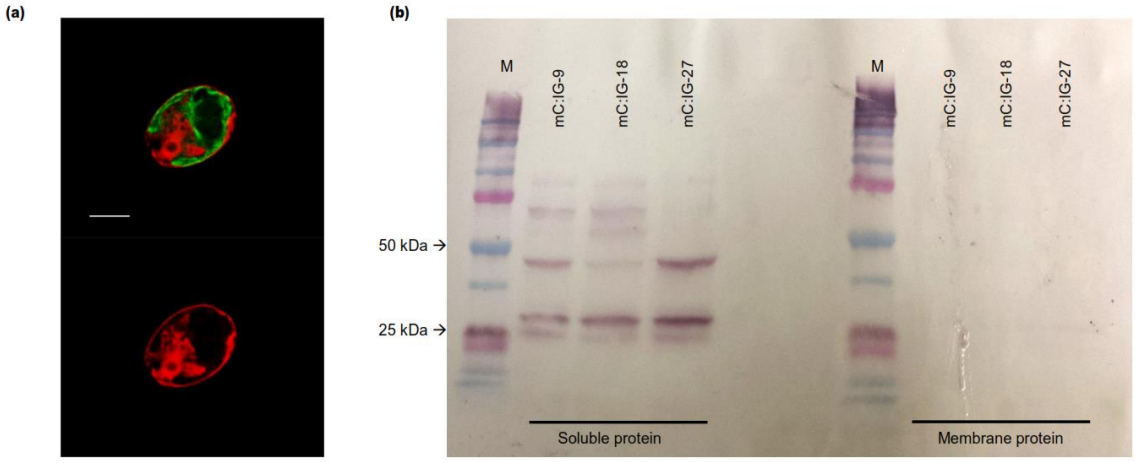

Figure 3.2 Fluorescence microscopy and western blot of mCherry:IG. A. Chlorophyll fluorescence (green) and mCherry fluorescence (red) of mCherry:IG fusion protein shown on top, mCherry:IG only below. B. Western blot of three mCherry:IG transformants showing soluble protein fractions on the left and membrane fractions on the right. Predicted sizes: mCherry 26kDa, IgASE1 29kDa, mCherry:IG 55kDa.

GC-MS

After the initial screening, 50mL cultures were grown in triplicate for both wild type CC1690 and the transformant. After 3 days of growth, GC-MS was performed on cell pellets of transformed and wild type CC1690 and the transformant IG16 showed accumulation of the lipids EDA and ETrA at 4.2 and 1.2% of total lipids, respectively (Fig 2A,B).

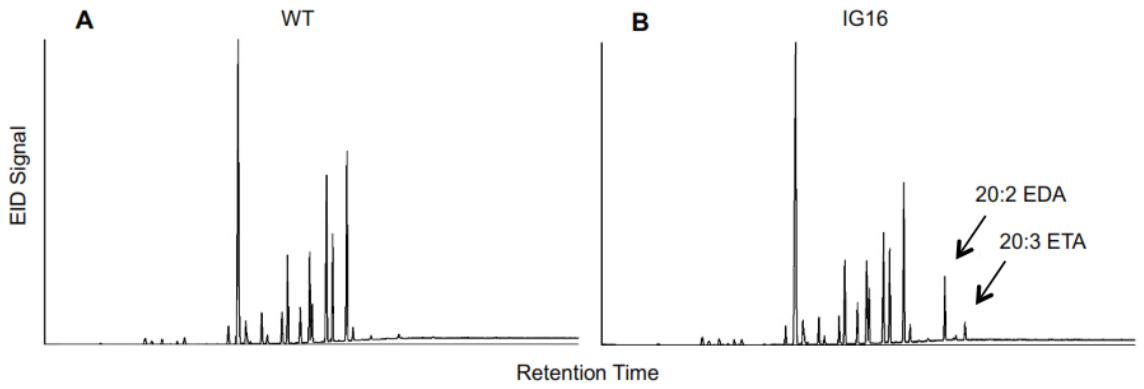

Figure 3.3 GC-MS. A. Wild type *C. reinhardtii* chromatogram after 72 hours growth in TAP. B. Transformant IG16 chromatogram after 72 hours growth in TAP. Arrows indicate EDA (left arrow) and ETrA (right arrow) production in transformed strain IG16.

RT-QPCR and EDA/ETrA Production

Four transformants were selected which showed a range of C20 production from .5% to 3% of total fatty acids in order to assess the impact of transgene expression on the conversion of lipid substrates to products (Fig 3). Cultures were grown in 50mL flasks in triplicate and RNA extracted and converted to cDNA on the same day as GCMS analysis was performed in order to closely link gene expression and lipid accumulation. There was a ~750-fold change in transcript abundance from the lowest expressing transcript to the highest, while there was only a ~6-fold change in lipid substrate conversion to products. Comparing the two lowest expressing transformants, IG74 and IG210, shows that up to a 15 fold change in transcript level can have little to no effect on the conversion of C18 substrates into C20 products. LCMS was performed to verify protein accumulation and revealed an ~8.7 fold increase in protein accumulation between IG210 and IG16 (Supp Fig 2). Elongase peptides were undetectable in the extremely low expression IG74 colony.

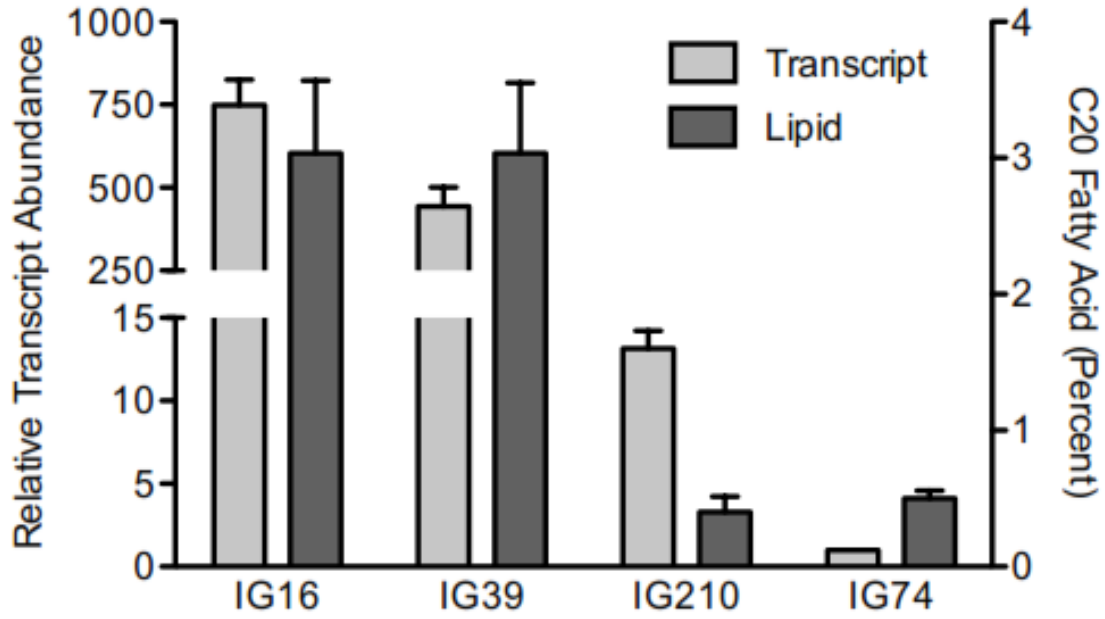

Figure 3.4 Transcript and lipid abundance of selected transformants. Transcript abundance (left y-axis) normalized to IG74 transformant and relative abundance of C20 fatty acids EDA and ETrA (right y-axis) for four transformed strains shown on x-axis. Error bars indicate mean ± standard error of the mean. RT-PCR data are from three technical replicates of three biological replicates, lipid data are from three biological replicates.

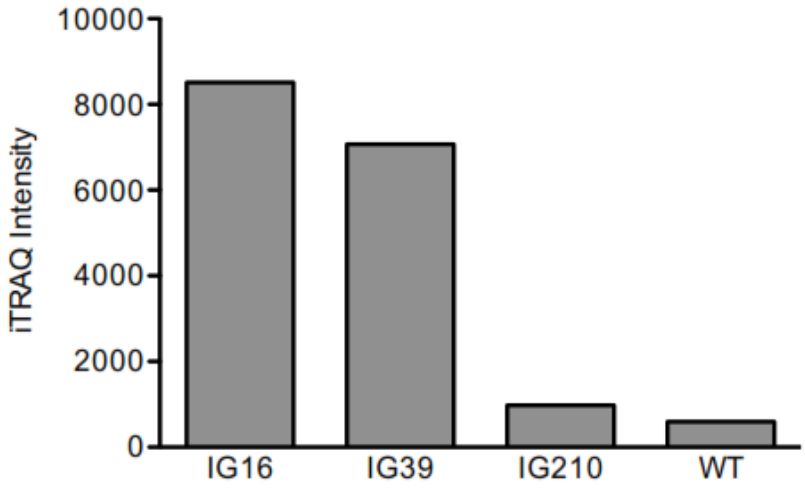

Figure 3.5 iTRAQ quantification of elongase protein accumulation. Signal intensity for iTRAQ labelled elongase peptides showing a comparison between wild type, IG16, IG39, and IG210.

Changes to Lipid Profile

The proportionality of total lipids remained largely unaffected by the elongase, and its effects were mostly limited to its substrates C18:2 n-6 and C18:3 n-3 which were the groups elongated to EDA and ETrA, respectively (Fig 4). The only unexpected and statistically significant change in lipid proportion came in the C18:1 n7 group, which nearly doubled in abundance in the transformant. By comparing the relative percentages of the substrate fatty acids C18:2 and C18:3 n3 to the products, we estimate that ~32% of the total C18:2 n6 was converted to EDA and ~8% of the C18:3 n3 pool was converted to ETrA.

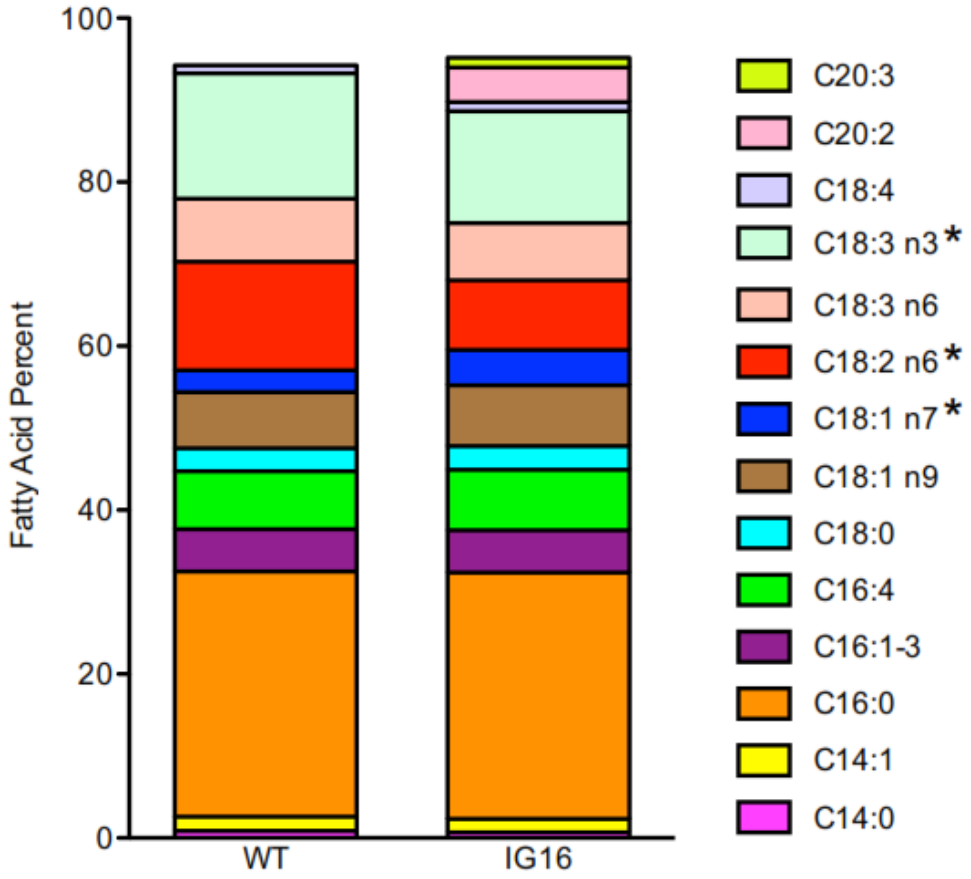

Figure 3.6 Wild-type and IG16 transformant lipid profile by GCMS. Percentage of total for the major lipids of *C. reinhardtii* comparing changes between wild type and IG16 from three biological replicates. Asterisks indicate statistically significant changes from three biological replicates analyzed with a student's t-test using $p<.05$.

TLC and Nitrogen Starvation

Thin layer chromatography and copper sulfate exposure were used to separate and visualize lipid groups based on their polarity using the system developed by Fan *et al*.[10] Groups of lipids were scraped off with a razor and transesterified to run separately by GCMS in order to identify which groups of lipids were affected by the expression of the elongase. Under nitrogen replete growth conditions, the phosphatidylethanolamine (PE) fraction accumulated C20 fatty acids at 1.2% of total PE. Upon 72 hours of nitrogen starvation, this shifted to only .01% of PE lipids being C20 fatty acids while the TAG fraction contained 4.7% C20 fatty acids. After 48 hours of nitrogen refeeding, levels of C20 increased in the ER to .12% C20 fatty acids and in TAG their proportion increased dramatically to 18.4% of lipids (Fig 5). The conditions of the nitrogen starvation and refeeding experiment were decided upon in order to maximize the rapid production and mobilization of TAG according to the work by Siaut *et al*.[17]

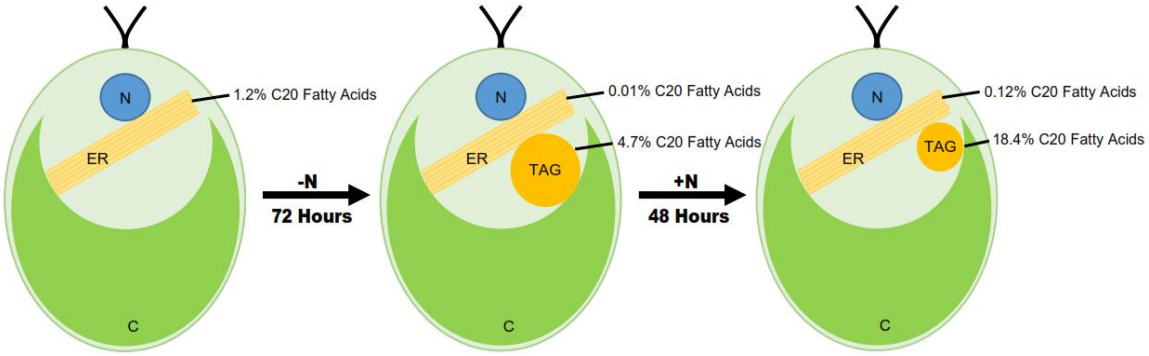

Figure 3.7 Localization of C20 after nitrogen deprivation and refeeding. Initial C20 fatty acid distribution in nitrogen replete conditions and changes in response to nitrogen deplete media for 72 hours and refeeding for 48 hours. N – nucleus, C – chloroplast, ER – Endoplasmic reticulum, TAG – Triacylglyceride.

Discussion

The *IgASE1* gene has been successfully expressed in *Chlamydomonas reinhardtii*. Consistent with previous studies of this enzyme, it appears to be limited to linoleic acid and *α*-

linolenic acid as substrates.[12, 18] EDA and ETrA have been detected at up to 4.2 and 1.2% of total fatty acids and there have been no apparent impacts to morphology or growth of the transgenic microalgae.

As metabolic engineering in microalgae moves forward, identifying limiting factors for high level conversion of substrates to products will be paramount. In this work, there was only a 1.6% success rate for the production of C20 fatty acids in gene positive transformed colonies. Along with the RT-PCR and LCMS data, this suggests that a crucial point of improvement for metabolic engineering of microalgae is still at the level of gene expression and mRNA stability. In order to improve substrate conversion, more robust expression systems will be required.

Producing C20 lipids in *C. reinhardtii* had the predicted effect of diminishing substrate pools as well as an unexpected effect on a non-substrate lipid. Increases in the ratio of C18:1 n7 may have been an indirect result of the decrease in abundance of the substrate lipids C18:2 n6 and C18:3 n3. In higher plants feedback loops have been discovered which compensate for decreases in lipid species by increasing fatty acid synthesis, and uncovering the reason for an increase in the relative proportion of C18:1 n7 will be an area of further study.[19]

The relatively lower conversion rate of C18:3 into ETrA may be due to its much lower prevalence in the phosphatidylethanolamine lipids of *C. reinhardtii*.[20] Although this likely has skewed the conversion rate down, it is important to note that the diacylglycerol-*N*,*N*,*N*-trimethylhomoserine (DGTS) lipids contain a high proportion of C18:3 n3 and show no accumulation of EDA or ETrA, indicating that the conversion efficiency is affected by the interplay of enzyme compatibility with the lipid group and availability of substrate lipids in the group.[21]

It is interesting to note that both PE and DGTS would be expected to incorporate C20 lipids, but this is not the case. Uncovering the reason EDA and ETrA are incorporated into PE and not DGTS will be an area of future focus, although the cause may be due to substrate specificity of the acyl-transferases expressed in the endoplasmic reticulum. It is likely that the elongase is acting

not on the diacylglycerol precursor of both PE and DGTS but after lipids have been shunted toward PE production.[22] Alternatively, it is possible that the elongase is incompatible with the DGTS lipid group itself and cannot interact directly with its acyl chains. The lack of C20 integration into the majority of *Chlamydomonas* lipids may be the result of incompatibility with ER and plastid lipases and acyltransferases, which have been shown to alter and incorporate particular lipid species in a site specific manner.[10, 23]

Nitrogen starvation revealed that C20 lipids are capable of being incorporated into TAG. On the other hand, the refed cultures' subsequent increase in C20 proportion suggests that C20 lipids may not be readily reincorporated into many of the lipid groups of *C. reinhardtii*. Co-expression of acyltransferases from LC-PUFA accumulating microalgae may be necessary in order to increase the incorporation of C20 lipids into endogenous lipid groups.

To the authors' knowledge, this work is the first successful demonstration of the use of metabolic engineering to produce C20 LC-PUFAs in *Chlamydomonas reinhardtii*. As the world continues to grow so does the demand for high value food products and the need for fuels. In order to meet either of these needs it will be necessary to develop a framework for metabolic engineering in high productivity organisms like microalgae to produce custom lipid profiles.

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Chapter 3, in full, is a reprint of the material as it has been prepared for submission. The dissertation author is the primary investigator and author of this paper. The other author on this paper is Stephen P. Mayfield.

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CHAPTER 4:

MOLECULAR TOOLS FOR TRANSFORMATION AND CHARACTERIZATION OF *CHLORELLA*

Abstract

Molecular tools for transformation and gene expression in *Chlorella* species are required to turn this production strain of algae into a tool for biotechnology and industry. Growth data and cell wall disruption assays were generated for a variety of species to identify top candidates for genome sequencing. Genome sequencing was performed on the *Chlorella vulgaris* UTEX 259 species and assembly resulted in a large amount of sequence data with which to build transformation constructs. Several constructs were cloned which allowed testing of a variety of transformation conditions. Although unsuccessful, these tools and techniques may prove fruitful to future researchers.

Introduction

The genus *Chlorella* contains single celled species of microalgae that have emerged as leading candidates to become production strains of microalgae.[1-3] They have long been used in Chinese aquaculture as a food source because they are a rich source of vitamins, minerals, polyunsaturated fats, and proteins.[4] The productivity and hardiness of this genus of microalgae has been a cause of interest for researchers for many years. *Chlorella* have high lipid content and have been looked at as a potential strain for the production of biofuels.[3] Because of this, development of a transformation system is paramount to the future of this species as an industrial biotechnology organism.

Unlike other microalgae such as *Chlamydomonas* and *Dunaliella*, *Chlorella* are surrounded by a thick outer cell wall similar to higher plants.[5] This wall has presumably led to much of the difficulty in achieving transformation of this genus of microalgae.[6] Another major barrier to transformation is the lack of sequence data available from which to construct transformation vectors. This work sought to characterize several species of *Chlorella,* identify the most promising species to have the genome sequenced, and then to construct transformation vectors

from the endogenous sequences of *Chlorella* and test various transformation protocols in the hopes of achieving stable transformation.

Results and Discussion

Strain selection

A collection of eight different isolates of *Chlorella* were obtained from the UTEX collection of algae. Five isolates of *Chlorella vulgaris* were examined as well as two isolates from *Chlorella sorokiniana* and one isolate from *Chlorella kessleri*. Growth assays were performed in HSM and TAP media in order to identify top strains and biomass productivity was assessed on a grams per liter basis (Fig 4.1 and Table 4.1, respectively). Because it was near the top in biomass productivity as well as easy to plate and culture in liquid, *Chlorella vulgaris* UTEX 259 was selected to move forward for genome sequencing.

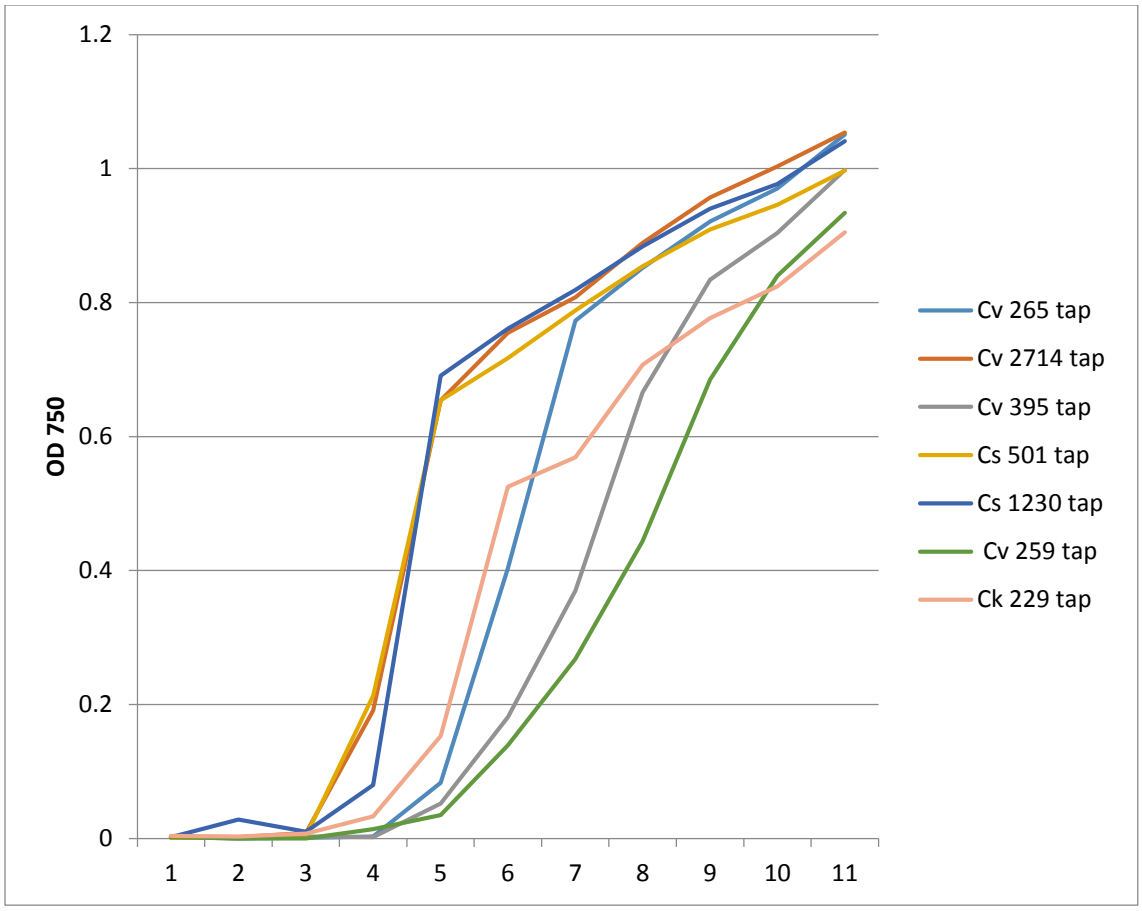

Figure 4.1 Growth of various *Chlorella* species in TAP media. Numbers on X-axis indicate days of growth post inoculation. Cultures were inoculated at 1*10^5 cells/mL. Cv- *Chlorella vulgaris*, Cs – *Chlorella sorokiniana*, Ck – *Chlorella kessleri*. Numbers refer to UTEX collection designations, i.e. Cv 259 – *Chlorella vulgaris* UTEX 259

Genome sequencing and assembly

After assessing biomass and growth profile, *Chlorella vulgaris* UTEX 259 was selected for genome sequencing. Using the Ion torrent sequencing platform and CLC assembly, the genome was sequenced and assembled. The size of the genome appeared to be 33 million base pairs based on sequence data and matched read length averaged 201 base pairs. The sequence was assembled into contigs averaging 3.2 kilobase pairs using the CLC assembly suite. Contigs were then BLAST searched against a collection of highly expressed genes from other microalgae including *Chlamydomonas reinhardtii* and *Scenedesmus dimorphus*. Genes used for BLAST searching in

contigs were: alpha/beta/epsilon/gamma tubulin, light harvesting complex, actin, desaturases, photosystem 1 subunit D, photosystem 2 subunit O, 60s ribosomal subunit, and rubisco small subunit. The BLAST-X tool allowed for comparison of genomic DNA from *Chlorella vulgaris* with protein sequence data from the aforementioned genes. Matches for the genes were identified and classified based on their coverage and level of matching. Importance was placed on identifying sequences which contained the beginning and end of the protein as the flanking sequences were the ultimate target of this portion of data analysis.

Table 4.1 Peak culture density and biomass production for *Chlorella* species. Strains and growth media shown as well as the peak cell count and wet biomass in grams per liter for each culture and condition. Cv- *Chlorella vulgaris*, Cs – *Chlorella sorokiniana*, Ck – *Chlorella kessleri*. Numbers refer to UTEX collection designations, i.e. Cv 259 – *Chlorella vulgaris* UTEX 259

Strain/Media	Cell Count (Cells/ml)	Wet Biomass (g/L)
Cv 265 tap	1.72*10^8	9.2
Cv 2714 tap	1.18*10^8	6.4
Cv 395 tap	1.51*10^8	10.4
Cs 501 tap	1.62*10^8	8
Cs 1230 tap	8.28*10^7	6.8
Cv 259 tap	1.46*10^8	8.8
Ck 229 tap	9.04*10^7	7
Cv 265 hsm	3.42*10^7	2.8
Cv 2714 hsm	7.85*10^7	3.2
Cv 395 hsm	1.15*10^8	2.4
Cs 501 hsm	5.81*10^7	2
Cs 1230 hsm	7.44*10^7	4.8
Cv 259 hsm	8.01*10^7	3.6
Ck 229 hsm	3.10*10^7	3.8

Construction of transformation vectors

Transformation vectors were designed using vectors built to transform other microalgae such as *Chlamydomonas reinhardtii* as a template.[7] The *sh-Ble* gene and the *aph7* genes were used as selectable markers providing resistance against Zeocin and Hygromycin, respectively. The resistance genes were codon optimized for *Chlamydomonas reinhardtii* but were found to be

suitable for use in *Chlorella vulgaris* because the nuclear codon bias of *Chlorella* was not significantly different from that of *Chlamydomonas.*

Because flanking sequences were required to serve as promoter/UTR regions for transformation construct building, only genes which had matches in BLAST-X searches and had coverage of both 5' and 3' coding regions were used to build *Chlorella* transformation vectors. The genes which matched with coverage of both 5' and 3' regions included: photosystem 1 subunit D, 60s ribosomal subunit, and rubisco small subunit. The majority of genes which were searched for in the *Chlorella* genome could not be matched with high enough confidence to determine the flanking regions.

After BLAST searching, flanking sequences were PCR amplified. Sequences ranging from 1500 base pairs upstream of the translation start site and 750 base pairs downstream of the translation stop site were chosen as flanking regions for vector construction. The goal of amplifying a larger upstream flanking region was to leave room for the presence of a promoter as well as a 5' UTR. Flanking regions were placed upstream and downstream of resistance genes and cloned into the pBR9 vector for amplification in *E. coli*.

Transformation attempts

Numerous strategies for transformation were employed, the majority of them based on techniques commonly used to transform other species of microalgae such as *Chlamydomonas reinhradtii*. Electroporation was the primary route used in attempting to deliver DNA as it has been the most widely successful for transforming microalgae thus far.[8] Alternate means of transformation included particle bombardment with gold nanoparticles and *Agrobacterium tumefaciens* mediated transformation. A summary of transformation attempts and results is shown in Table 4.2. Other transformation constructs were tried as well including the Cauliflower mosaic virus 35S promoter (CaMV 35S) and the AR1 *C. reinhardtii* nuclear promoter with both *sh-Ble*

and *Aph7* as resistance genes because of their success in transforming *Chlamydomonas*.[9] Although there were a wide variety of transformation attempts made, no successfully transformed colonies appeared on selection plates.

Table 4.2 Transformation conditions tested. These transformation conditions were tried for all strains of *Chlorella* discussed above.

Condition	Volts per Cm	Construct	Result
4mm Cuvette 40mM TAP sucrose	**1500-2500v/cm**	**60s Hyg, 60s Ble, PsaD Hyg, PsaD Ble, Rbcs Hyg, Rbcs Ble, CaMV35s**	**Negative**
4mm Cuvette 40mM TAP sucrose Cellulase treated	**1500-2500v/cm**	**60s Hyg, 60sBle, Rbcs Hyg, Rbcs Ble, PsaD Hyg, PsaD Ble**	**Negative**
2mm cuvette 40mm TAP Sucrose	**2000-14000v/cm**	**60s Hyg, PsaD Hyg, Rbcs Hyg, Rbcs Ble, 60s Ble, PsaD Ble**	**Negative**
2mm cuvette 5M sorbitol 50% glycerol	**2000-14000v/cm**	**60s Hyg, 60s Ble, PsaD Hyg, PsaD Ble, Rbcs Hyg, Rbcs Ble**	**Negative**
1mm cuvette .5M sucrose HEPES buffer	**10000-17500v/cm**	**60s Hyg, 60s Ble, PsaD Ble, RbcS Ble, RbcS Hyg, PsaD Hyg**	**Negative**
Agrobacterium	**NA**	**Chlamy Hyg vector**	**Negative**
Particle Bombardment 550d Gold/1000d Gold, 1350 psi	**NA**	**60s Hyg, 60sBle, Rbcs Hyg, Rbcs Ble, PsaD Hyg, PsaD Ble**	**Negative**

Enzymatic cell wall digestion

The rigid cell wall of *Chlorella* appeared to be preventing the ingress of DNA during transformation. After consulting literature, a selection of cocktails of enzymes which were used in order to assess the cell wall components of various species of *Chlorella* was identified.[10] By using enzymes from the paper and developing a screen to test their efficacy (Figure 4.2), we were able to strip some of the components from the cell walls of *Chlorella.* The assay relies upon the plasma membrane being exposed in cells stripped of their walls which results in the increase in fluorescence of a lipophilic dye in solution. This rapid test can be used for evaluating potential production strains of microalgae in the future in order to gain insight into the composition of their cell walls or to try to prepare them for transformation. The assay proved successful in that the cells with the highest fluorescence signal in the media had their cell walls visibly disturbed when examined under visible light microscopy. Unfortunately, when combined with transformation attempts, no colonies were attained by this method.

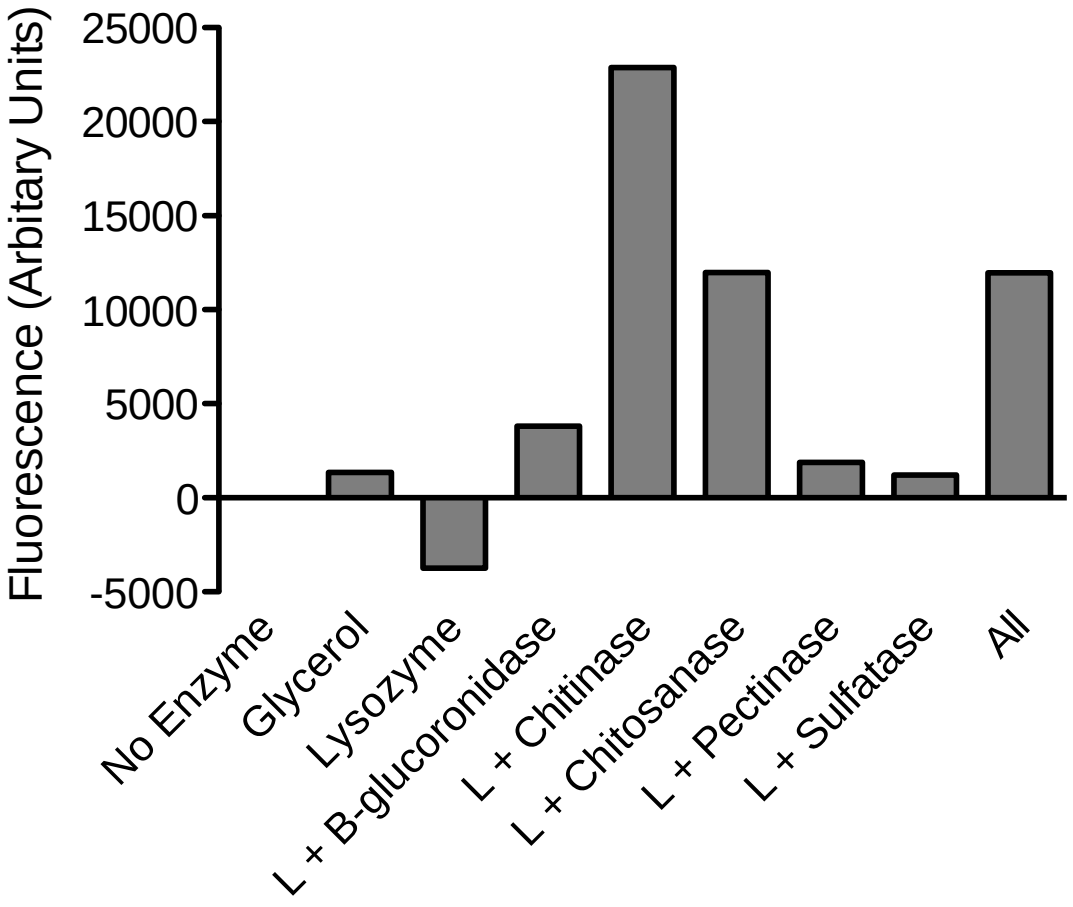

Figure 4.2 Cell wall digestion assay. Example of treated *Chlorella vulgaris* UTEX 259 cells shown. L – lysozyme treatment, all – lysozyme, B-glucoronidase, chitinase, chitosanase, pectinase, sulfatase.

Materials and Methods

Strains and growth conditions

Samples of *C. vulgaris* UTEX 259, 265, 395, 2714 and *C. sorokiniana* UTEX 2714 and *C. keslleri* UTEX 2229 were obtained from the Culture Collection of Algae at the University of Texas at Austin. *C. sorokiniana* CS-01 was provided by the Burkart lab at University of California at San Diego. All algae strains were inoculated in 50mL flasks of Sueoka's high-salt medium (HSM) or Tris-acetate-phosphate (TAP) media and grown at 25 °C under continuous illumination at

160 μmol m^{-2} s^{-1}. Cultures were shaken at 250 rpm for under continuous illumination (160 μmol m^{-2} s^{-1}) at 25 °C. Wet biomass for growth assays was assayed by spinning cells at 10,000 RCF and aspirating the supernatant twice, then using subtractive weighing to determine the grams of wet biomass.

Genome sequencing and assembly

Genomic DNA was prepared from *C. vulgaris* UTEX 259 using phenol-chloroform extraction and then treated with RNAse. Samples were sent for Ion Torrent sequencing and sequencing reads were assembled using CLC assembly. Scaffolds were searched for hits using the NCBI website's BLAST-x tool to identify matches.

Transformation vectors and transformations

Vectors were PCR amplified from *C. vulgaris* UTEX 259 to obtain flanking sequences for cloning into the pBR9 *Chlamydomonas reinhardtii* transformation vector backbone. Unless noted otherwise in Table 4.2, all transformation conditions were performed as described previously for *Chlamydomonas reinhardtii*.

Enzymatic Treatment

Cells were incubated with enzyme cocktails as indicated in TAP media with .5M sucrose for periods of 1, 6, and 12 hours. A sample of enzymatically treated cells was inoculated on drug free plates for each set of conditions and incubation times to ensure that the cells were still viable after enzymatic treatment.

Conclusion

The genus *Chlorella* is full of microalgae with remarkable capacity for robust growth and hardiness, making them a true species of interest for industrial biotechnology. The transformation protocols and vectors produced herein were unable to yield fruitful results, but they may inform the successes of other researchers to come. As sequence data becomes more available, new vectors may be built which enable transformation of *Chlorella* and will open up a new avenue of biotechnological research and real world value as a biomanufacturing platform. A rapid assay to detect cell wall disruption by enzymatic digestion should also help researchers bioprospecting a variety of species of microalgae as a means of roughly assessing their cell wall composition as well as potentially aiding in transformation of new species.

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CONCLUSION

Microalgae can truly become a transformative force in the economy, and a major source of environmentally friendly food and fuels due to their rapid growth, high biomass productivity per acre, and ability to be used as protein and lipid factories. Tools to enable more optimal cultivation, metabolic engineering of lipid profiles, and techniques to enable transformation of newly identified species will be paramount to the future of this research.

The research described herein will have some immediate effects on the state of the microalgae industry. In chapter 2, the development of rapid assays for growth monitoring as well as lipid and protein assessments will enable farmers to save money and to use less nutrients and maximize their yields, making microalgae cultivation more effective and more affordable in a commercial setting. In the laboratory, work from chapter 3 will provide a template for the modification of lipid metabolism in both *Chlamydomonas* and whatever species of microalgae become model organisms in the future. The questions created by transformation of lipid profile in *C. reinhardtii* will lead to more research into the nature of lipid compatibility with endogenous acyltransferases and likely will also cause researchers to question the nature of TAG reincorporation after nutrient refeeding. The work performed in chapter 4 should help researchers seeking to transform new species of microalgae by providing a roadmap for them to follow, and a new assay to use in the assessment of cell wall components.

As a result of this research, tools for both commercial algae cultivation and biotechnology research have been improved. Ease of cultivation will enable more people to try algae farming and as a result awareness and research will follow. There is no route forward for our society without some form of fungible alternative fuel and some form of low environmental impact nutrient source. The steps to metabolically engineer lipid profile demonstrated here can be adapted and altered for the production of both biofuels and nutrient rich foods for the future. With these tools, the future of microalgae biotechnology is certainly brightened.