Optimizing the Electroporation of *Ettlia oleoabundans* Protoplasts

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Abstract

Microalgae have the potential to be an effective feedstock for biofuels due to their rapid growth and production of biofuel precursors. The ability to express foreign genes within species of algae of interest for biofuel production can lead to a further understanding of how and why these species produce the lipid and hydrocarbon products that can be converted to fuel. The species *Ettlia oleoabundans* has been studied for its high lipid production and growth rates, suggesting its use as a feedstock for biodiesel production. No molecular research has been performed on *E. oleoabundans.* In this project protoplast electroporation was attempted. The cell wall of *E. oleoabundans* was degraded in an enzyme mixture of cellulase, pectinase, and macerase. Originally low protoplasting efficiencies, survival and growth were improved through the addition of 0.4M D-sorbitol and elimination of a transfer step by using PBS + sorbitol as the protoplasting medium. The development and maintenance of viable protoplasts opens the door to attempting several other transformation procedures.

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Chapter 1: Introduction and Background

Since the 1950's, a growing population and a push for industrialization has increased the global oil demand from 11 million barrels per day (MBD) to an estimated 80 MBD (Wright, 2008). It is predicted that by the year 2030 the global demand on oil will reach 115 MBD, likely straining the ability of global oil reserves to produce enough to meet the demand (Jackson, 2009). It is estimated that we may already be at our peak oil production, making it difficult to meet the ever growing demand for fossil fuels without an alternative source added to the supply (Kerr, 2011). A renewable energy source would have the benefit of filling some of the global demand while being beneficial to the environment and the general well-being of humans. Microalgae offer a promising source of renewable fuels which may be able to fill the void left by conventional oil production.

1.1 Benefits of Biofuels

Biofuels, which are fuels derived from a biological feed stock, are an alternative energy source attracting great attention. Biofuels have many potential benefits, ranging from improved human health to an improved environment.

1.1.1 Human Health Benefits

Biofuels of all types show significant promise in reducing damage to the environment currently caused by fossil fuels. Fuels combusted by automobiles represent a substantial source of many serious air pollutants. The harmful compounds in this pollutant include carbon monoxide, mutagenic hydrocarbons, and carcinogenic aromatics (Table 1) (Liaquat, *et al.,* 2010).

1.1.2 Environmental Benefits

Bio-synthesized fuels contain significantly fewer of these harmful compounds, so it is expected that their use will drastically reduce the amount of pollution released into the environment. Biodiesel and ethanol are the leading biofuels currently used, and even in limited amounts have resulted in a significant decrease in harmful emissions (Liaquat, *et al.,* 2010). The emissions released by traditional fossil fuels in automobiles have many detrimental effects to the environment but also on animals including lead poisoning, lung damage from hydrocarbon air pollution, and many forms of cancer from sources such as formaldehyde and polycyclic aromatic hydrocarbons (Liaquat, *et al.,* 2010). Biofuels significantly reduced emissions when blended with conventional diesel and gasoline automotive fuels. (Liaquat, *et al.,* 2010). Blends of biodiesel with petroleum diesel have reduced the fumes, odor, and harmful emissions produced by vehicles typically powered by the diesel fossil fuels alone (Liaquat, *et al.,* 2010). Further reduction of these emissions will protect and improve our environment, safety, and quality of life.

Table 1: Motor vehicle emissions and their human health effects (taken from Liaquat *et al.*, 2010)

Exhaust emissions	Health Effects
Carbon Monoxide	Impairs perception and thinking, slows reflexes, causes drowsiness, brings on angina, and can cause unconsciousness and death; it affects fetal growth in pregnant women and tissue development of young children. It has a synergistic action with other pollutants to promote morbidity in people with respiratory or circulatory problems
Nitrogen Oxides (NO_2, NO_3)	Can increase susceptibility to viral infections such as influenza; irritate the lungs and cause edema, bronchitis and pneumonia; and result in increased sensitivity to dust and pollen in asthmatics. Most serious health effects are in combination with other air pollutants
Hydrocarbons and other Volatile Organic Compounds	Low-molecular weight compounds: Eye irritation, coughing and sneezing, drowsiness and symptoms akin to drunkenness. Heavy molecular weight compounds: may have carcinogenic or mutagenic effects. Some hydrocarbons have a close affinity for diesel particulates and may contribute to lung disease
Ozone (Precursors: HC & NOx)	Causing coughing, choking, and impaired lung function; causes headaches and physical discomfort; reduces resistance to colds and pneumonia; can aggravate chronic heart disease, asthma, bronchitis, and emphysema
Lead	Affects circulatory, productivity nervous, and kidney systems suspected of causing hyperactivity and lowered learning ability in children; hazards even after exposure
Particulate Matter (PM)	Respiratory problems, lung cancer and cardiopulmonary deaths
Toxic Substances	Causing cancer, reproductive problems, and birth defects. Benzene and asbestos are known carcinogens; aldehydes and ketones irritate the eyes, cause short-term respiratory and skin irritation and may be carcinogenic
Polycyclic aromatic hydrocarbons (PAHs)	Lung cancer
Formaldehyde	Eye and nose irritation, coughing, nausea and shortness of breath. Occupational exposure is associated with risk of cancer
Dioxin	Long-term exposure: Impairment of the immune system, the developing nervous system, the endocrine system and reproductive functions

1.1.3 Economic Benefits

The production of biofuels today requires a large investment in the initial startup of a production facility, and is not yet competitive with conventional fuels; however, there are still socio-economic benefits from producing biofuels (Rutz and Janssen, 2007). Biofuel production can provide new domestic jobs while opening the door to small businesses in the exclusive oil industry. Furthermore, growing biofuel feedstocks could benefit farmers by adding an additional source of income for their crops (Rutz and Janssen, 2007).

The cost of crude oil has been steadily rising for the past decade (Senauer, 2008). The impact of this increase can be seen in other commodities as their prices have increased proportionally to the cost of oil (Figure 1).

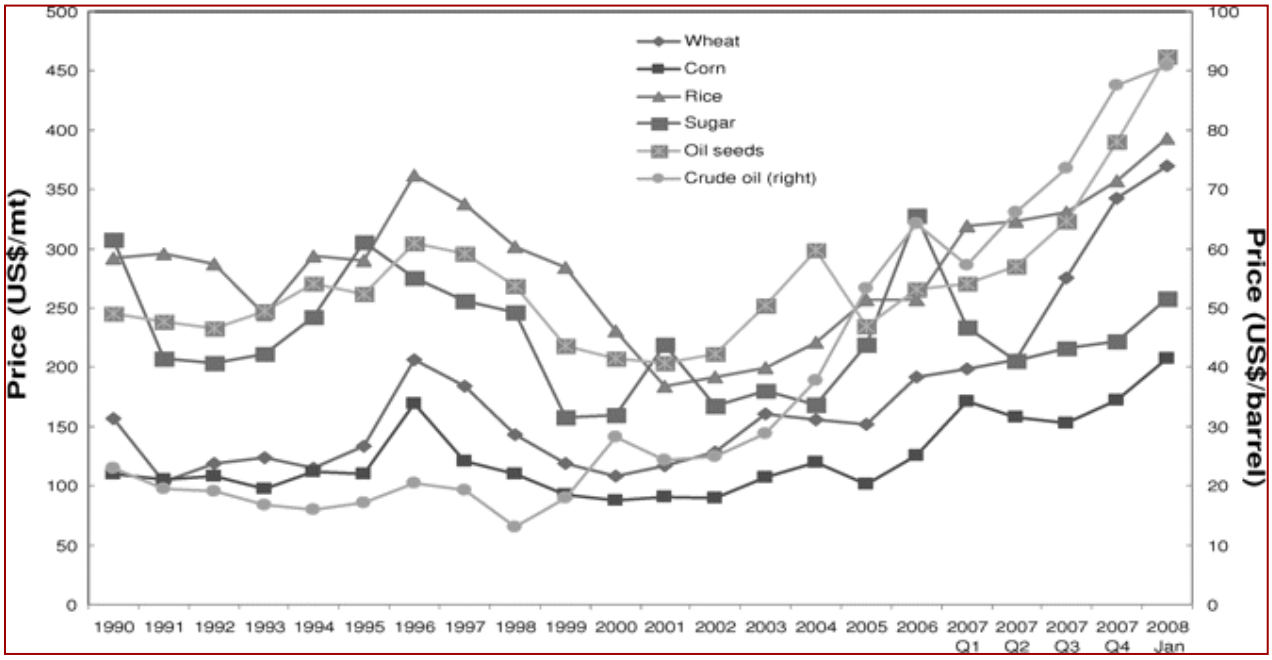


Figure 1: Worldwide commodity prices compared to the cost of crude oil per barrel from 1990-2008 (taken from Senauer, 2008).

1.2 Microalgae as a Biofuel Source

An alternative energy source must be able to compete with conventional fuel prices by being renewable and having low production costs. A biological feedstock must be able to grow quickly with few, low cost nutrient inputs, so that the energy output of the fuel is greater than the energy required for the entire process lifecycle to produce the biofuel. Crops such as corn and soybeans have been the major feedstock explored for biofuel production; however, microalgae have been explored since the 1970s for their promise of being a more efficient source of energy.

1.2.1 Microalgae Growth Requirements

Microalgae are relatively low maintenance organisms requiring little input of nutrients for growth. Microalgal cultures are able to grow and survive when supplied with light, water (H_2O), carbon dioxide (CO_2), and inorganic nutrients such as nitrates, phosphates, iron and some trace elements (Chisti, 2008). A media containing these nutrients can provide microalgae with the materials to produce compounds of importance, such as the precursors to biofuels.

1.2.2 Microalgae Produce Two Types of Oils

There are two types of high energy oil products produced by algae, lipids in the form of triacylglycerol (TAG) and hydrocarbons produced through the terpene biosynthesis pathway (Gouveia *et al.,* 2009, Metzger and Largeau, 2005). These two oil products are quite different in

their chemical properties and the way they can eventually be used as fuels. Lipids are typically extracted and converted to biodiesel while hydrocarbons could possibly be added directly into a conventional oil refining process as they are very similar in structure to crude oil (Gouveia *et al.,* 2009, Metzger and Largeau, 2005).

1.2.3 Biochemistry of Triacylglycerol Production

CO_2 is metabolized through photosynthesis, and the energy may be stored inside microalgae in the form of lipids. Although the lipid biosynthetic pathway has not yet been completely determined for microalgae, studies have shown that microalgal pathways are similar to plants. The lipid biosynthetic pathway in plants begins in a plant organelle, the chloroplast, and yields fatty acids (Figure 2). Fatty acid synthesis starts with acetyl-CoA, which is converted to malonyl-CoA by the addition of CO_2 via acetyl-Co A carboxylase. Malonyl –CoA then then associates with acyl carrier protein (ACP) by the enzyme malonyl CoA ACP transacylase to yield malonyl-ACP. Malonyl-ACP is then combined with another acetyl-CoA molecule through decarboxylation by fatty acid synthase to form acetoacyl-ACP. This product then has its keto group removed in 3 steps by 3-keto acyl ACP rductase, 3-hydroxy acyl ACP rductase and enoyl ACP reductase to produce butyryl-ACP, which is then combined with another malonyl-ACP on fatty acid synthase, releasing CO_2, forming a 6-carbon product. This product then undergoes further condensation reactions with malonyl-ACP, each time adding two carbons to the overall molecule until a fatty acid chain of 16 or 18 carbons is produced (Figure 2).

The fatty acids undergo further modification in the cytoplasm and plant endoplasmic reticulum (ER), resulting in TAGs. TAGs are extracted from the cell and converted to biodiesel. In the cytoplasm double bonds are added to the carbon chains by fatty acid desaturases. These double bonds desaturate the product of hydrogen atoms, making it less stable when converted to biodiesel. The Kennedy Pathway (Figure 3) modifies lipids passing through the ER by linking the fatty acids one at a time with ester bonds to glycerol molecules (Figure 4) (Lung and Weselake, 2006). The first fatty acid is added to glycerol-3-phosphate by glycerol-3-phosphate acetyltransferase, yielding lysophosphatidic acid. An additional fatty acid is placed on the glycerol backbone by lysophosphatidic acid acetyltransferas. The produced phosphaditic acid molecule has the phosphate on the third carbon removed by phosphaditic acid phophatase, yielding diacylglycerol, containing a hydroxyl group on the third carbon. The final fatty acid is added by diacylglycerol acetyltransferase producing triacylglycerol (Figure 3). In summary, three fatty acids are added per one glycerol molecule, yielding a TAG (Figure 5).

The TAGs generated in the ER accumulate and break off as oil bodies (Figure 6). Oil bodies have single layered phospholipid membranes, which keep the TAGs enclosed (Taiz and Zeiger, 2008). Membrane proteins called oleosins prevent the accumulated oil bodies from interacting with one another (Taiz and Zeiger, 2008). The TAG accumulation and oil body formation in algae is likely the same mechanisms as in plants.

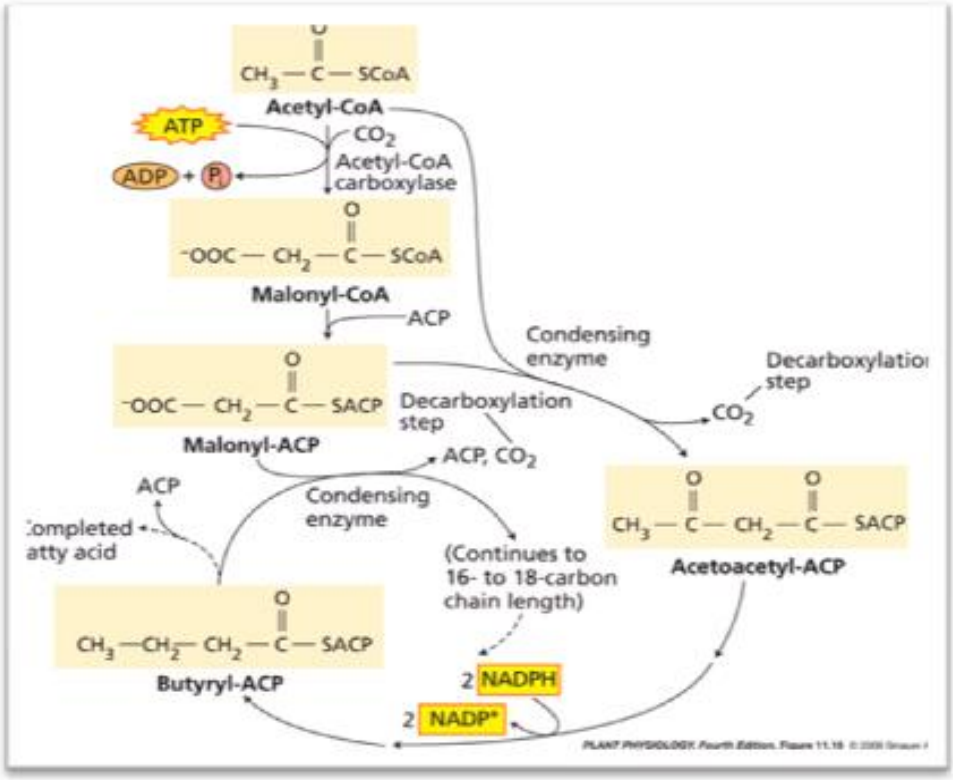


Figure 2: Lipid Synthesis in Plant Plastids (taken from Taiz and Zeiger, 2008)

Many microalgal species accumulate high levels of TAGs during their stationary growth phase. The species *Ettlia oleoabundans* has been reported to produce up to 56% of its dry weight in TAGs when starved for nitrogen, and therefore growing at a very slow rate (Gouveia *et al.,* 2009). Microalgae's ability to produce and store such high volumes of TAGs makes them prime candidates for a renewable energy source. Compared to other biological feedstocks such as soybeans and cotton, microalgae have many advantages as a sustainable renewable energy source (Table 2). TAG producing microalgae require considerably less land for higher energy yield than any biological feedstock currently used for biofuel (Table 2) (Schenk *et al.,* 2008).

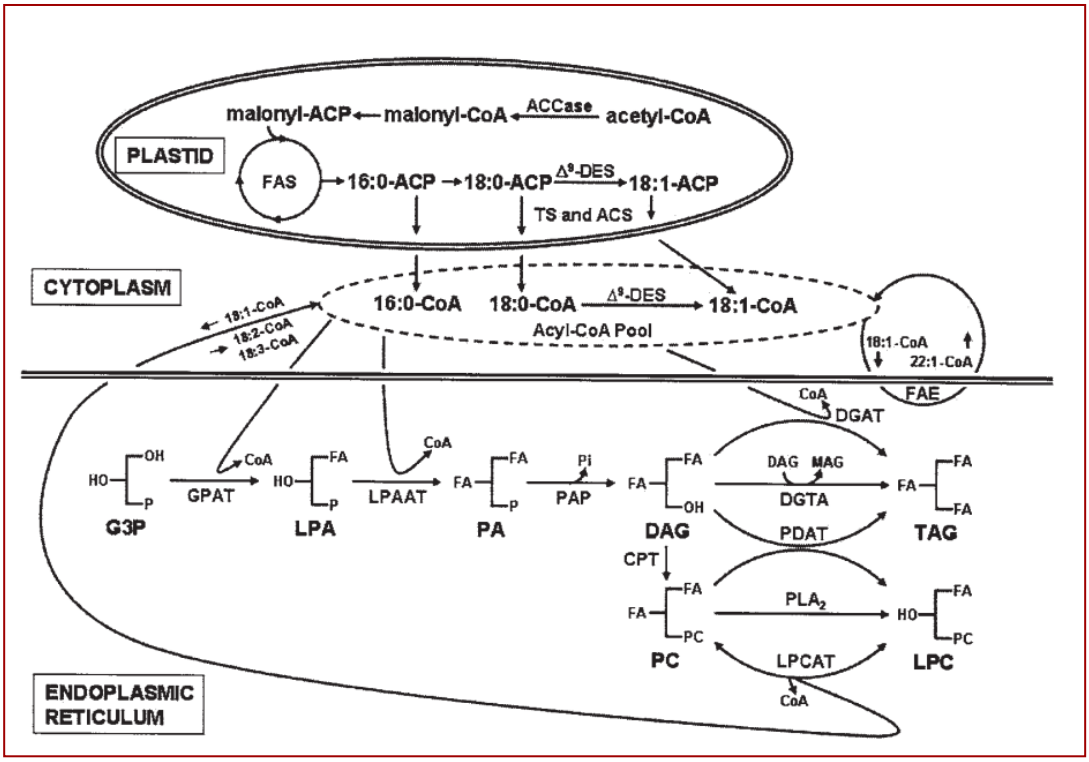


Figure 3: The Kennedy pathway adds three FAs per glycerol molecule, forming a TAG (taken from Lung and Weselake, 2006).

Abbreviations: ACCase- acetyl CoA carboxylase; FAS- fatty acid synthesis; G3P-Glycerol-3-phosphate; GPAT-Glycerol-3-phosphate acyltransferase; LPA-Lysophosphatidic acid; LPAAT-Lysophosphatidic acid acyltransferase; PA-Phosphatidic acid; PAP-phosphaditic acid phosphatase; DAG- diacylglycerol; PDAT- phospholipid diacylglycerol acetyltransferase; DGTA- diacylglycerol acetyltransferase; PC- phosphatidylcholine; LPC- lyso-phosphatidylcholine; LPCAT- lyso-phosphatidylcholine acetyltransferase; TAG- triacylglycerol)

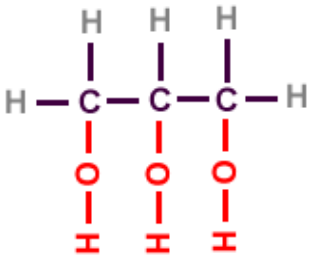


Figure 4: Glycerol molecule which forms the backbone of a TAG (taken from International School of Caracas, 2011).

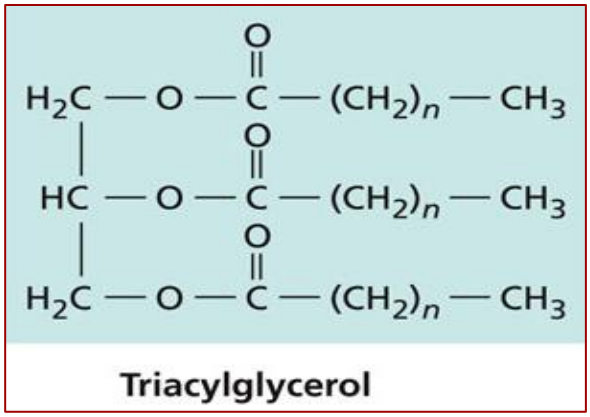


Figure 5: Triacylglycerols are produced in plants and similarly in algae, and are used for biodiesel production (taken from Taiz and Zeiger, 2008).

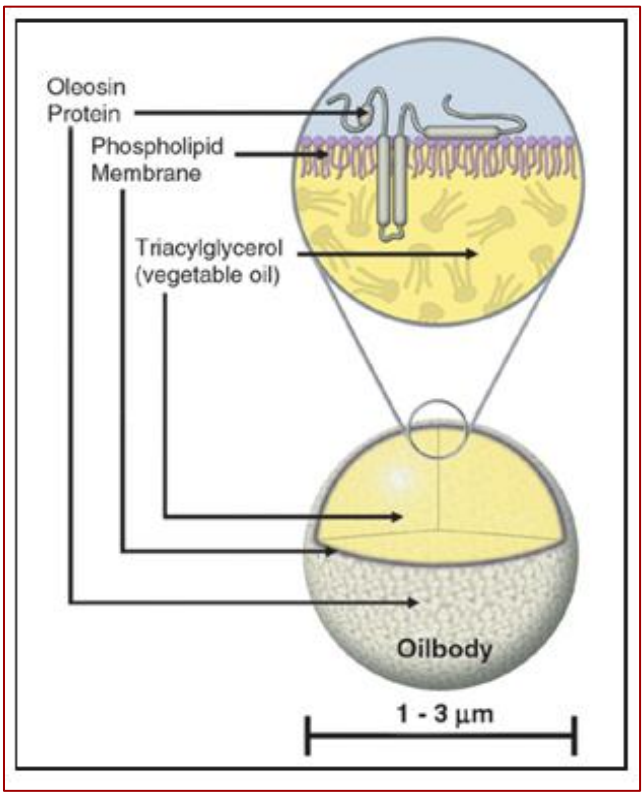


Figure 6: Oil bodies formed in plants store TAGs (taken from Taiz and Zeiger, 2008).

Table 2: Comparison of oil production and land requirements between various crops considered for biodiesel production (taken from Schenk *et al.,* 2008).

Plant Source	Biodiesel (L/ha/year)	Area to Produce global oil demand (hectares x 10^6)	Area required as percent global land mass	Area as percent global arable land
Cotton	325	15, 002	100.7	756.9
Soybean	446	10, 932	73.4	551.6
Mustard Seed	572	8,524	57.2	430.1
Sunflower	952	5,121	34.4	258.4
Rapeseed/Canola	1,190	7,097	27.5	206.7
Jatropha	1,892	2,577	17.3	130
Oil Palm	5,950	819	5.5	41.3
Algae (10m-2 day-1 at 30% TAG	12,000	406	2.7	20.5
Algae (10m-2 day-1 at 50% TAG	98,500	49	0.3	2.5

1.2.4 Biochemistry of Hydrocarbon Production

Botyrococcus braunii produces long chain hydrocarbons (Metzger and Largeau, 2005). The hydrocarbons produced by *Botryococcus braunii* are called botryococcenes. Unlike the lipids of most plants and algae, the long chain hydrocarbons (LCH) produced by the Race B of *B. braunii* are formed by the terpene biosynthetic pathway. Radio labeling experiments have determined that the mevalonate (MVP) pathway is not used for precursor production of botryococcenes, rather the plastid localized methylerythritol phosphate (MEP) pathway is used (Metzger and Largeau, 2005). As in plants, the precursors used to produce these botryococcenes are produced from the MEP pathway are the five carbon isomers isopentenyl diphosphate (IPP) and dimethyallyl diphosphate (DMAPP) (Taiz and Zeiger, 2008). Three of these precursors are added together until a chain of 15 carbons is produced called farnesyl diphosphate (FPP), at which time two FPPs are joined to obtain a 30 carbon product, or a triterpene (Figure 7). This 30 carbon product is then modified to produce the other botryococcenes (Metzger and Largeau, 2005).

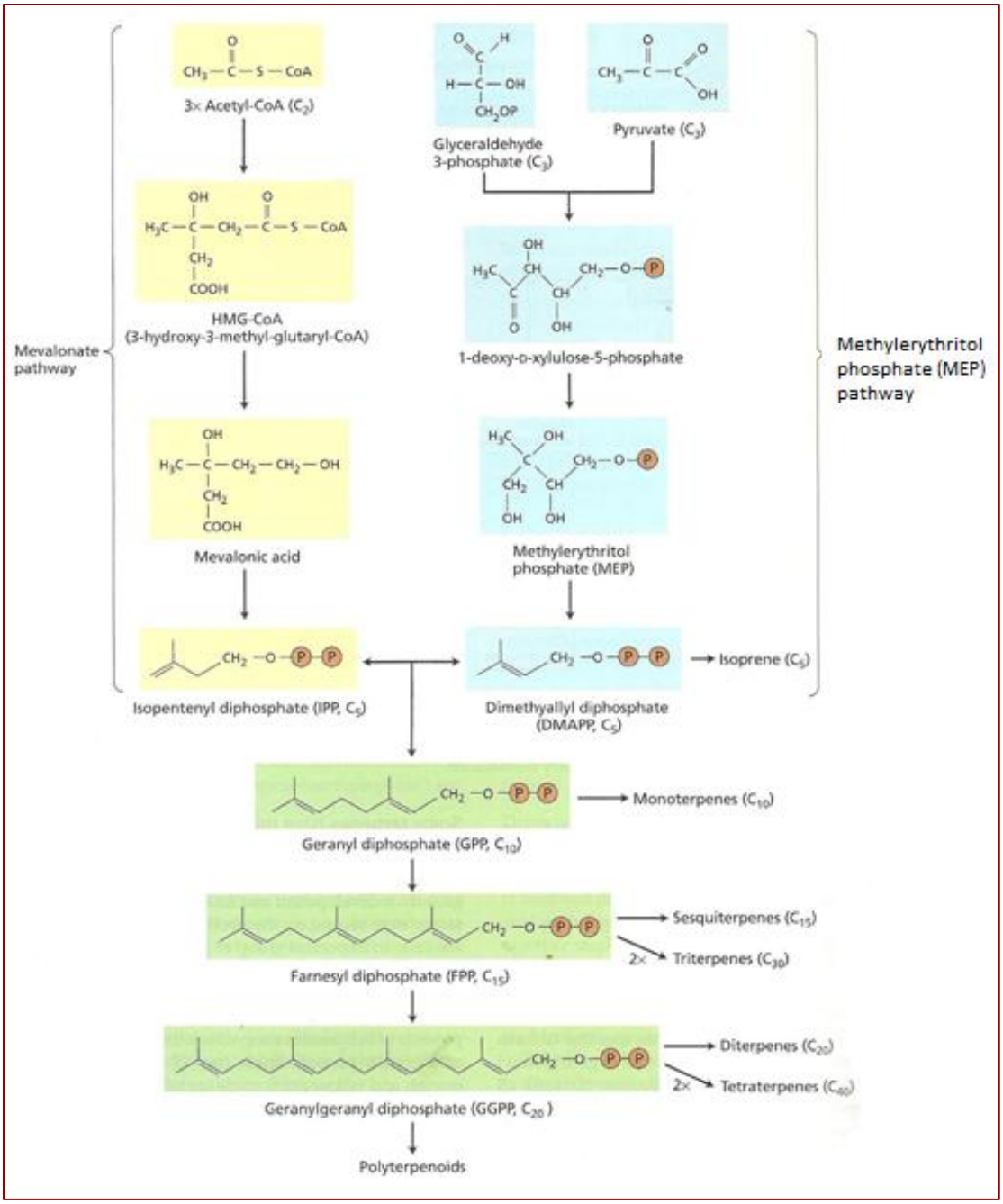


Figure 7: Terpene biosynthesis in plants (Taiz and Zeiger, 2008)

1.3 Algal Candidate for Biodiesell Production, *Ettlia oleoabundans*

Ettlia oleoabundans, formally known as *Neochloris oleoabundans* (Figure 8), is a unicellular freshwater green microalga originally isolated from sand dunes in Saudi Arabia (UTEX, 2010). *E. oleoabundans* can produce up to 56% of its dry mass in TAGs. The lipid

composition of the TAGs produced by *E. oleoabundans* is ideal for biodiesel production (Gouveia *et al.,* 2009). The major fatty acid produced is oleic acid (18:1), an unsaturated fatty acid with one double bond that is preferred for biodiesel as it has a low oxidation rate. Also, the alga accumulated only about 12% of the less desired linolenic acid (18:3), which contains three double bonds. Overall the oils in *E. oleoabundans* meet requirements of the European Standard EN for biodiesel (Gouveia *et al.,* 2009).

Additionally, the lipids produced by *E. oleoabundans* have an iodine level of 72, well below the maximum allowed value of 120 (Gouveia *et al.,* 2009). The iodine level is an indicator of the level of unsaturation in a sample of fuel. Iodine will react with the double bonds in biodiesel, so the iodine level is a measure of iodine reacted with 100g of a given sample (Schober and Mittelbach, 2007). If a sample of fuel has an iodine level above 120 it is considered to be less stable and, therefore, less desirable of a product as it is more susceptible to degradation via oxidation (Schober and Mittelbach, 2007).

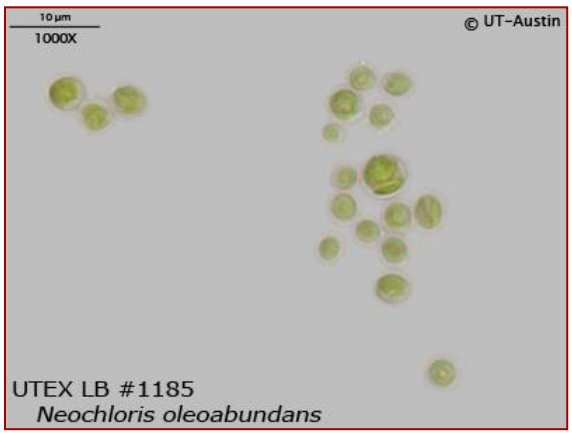


Figure 8: Microalgae *E. oleoabundans* (taken from UTEX, 2010).

When considering other algae of interest for production of biodiesel, the productivity of *E. oleoabundans* is comparable. *E. oleoabundans* is capable of a very high lipid content when conditions are optimal (nitrogen starvation); this is not significantly surpassed by other algal species (Gouveia *et al.,* 2009). *Ettlia* has a fast growth rate, but may be a bit slower than other species. For example, comparing different algal species, it was found that under the same growth conditions, *E. oleoabundans* achieved a growth rate of 0.09gDW/L/day and a maximum biomass concentration of 2.0gDW/L (Gouveia and Oliveira, 2008). In contrast, *Spirulina maxima* was able to grow at 0.2gDW/L/day to a maximum concentration of 3.1gDW/L. *Dunaliella tertiolectus* had a growth rate of 0.12gDW/L/day, and a maximum concentration of 3.6gDW/L (Gouveia and Oliveira, 2008). Although Gouveia *et al*. (2009) was able to increase growth rate and biomass yield of *E. oleoabundans* by efforts to optimize growth conditions, *E.*

oleoabundans still did not produce as much biomass as either *Spirulina maxima or Dunaliella tertiolectus* (Gouveia and Oliveira, 2008).

Gouveia *et al*. (2009) attempted to determine some of the optimal growth conditions required for producing biodiesel from cultured *E. oleoabundans*. They varied temperature, nitrogen, and CO_2 supplementation for a growth period of 18 days. Under the optimal growth conditions, *E. oleoabundans,* like many other algal species, does not produce a high percentage of lipids. Indeed, under these conditions *E. oleoabundans* has never been reported to produce more than 30% gTAGS/gDW, though lipid production rates were still high (37.6mg/liter day) (Gouveia *et al.,* 2009). While 30^{o}C produced the highest growth rates for *E. oleoabundans*, the highest dry weight was achieved at 26^{o}C producing around 1.6gDW/L. Under nitrogen depleted conditions, the biomass productivity was greatly decreased. The doubling time *for E. oleoabundans* increased tenfold after five days of growth for the nitrogen depleted cultures as compared to the cultures with ample nitrogen supply (Gouveia *et al.* 2009). However, when nitrogen starved, *E. oleoabundans* accumulated 56% gTAGs/gDW, double the concentration under the same parameters for optimal growth with nitrogen in the media (Gouveia *et al.,* 2009). When stained with Nile red, *E. oleoabundans* cells show very little lipid body staining during the logarithmic growth phase (Figure 9, A) when compared to cells that have been in the stationary growth phase for a week after nitrogen depletion (Figure 9, B) these cells are also much smaller than those growing in nitrogen replete media.

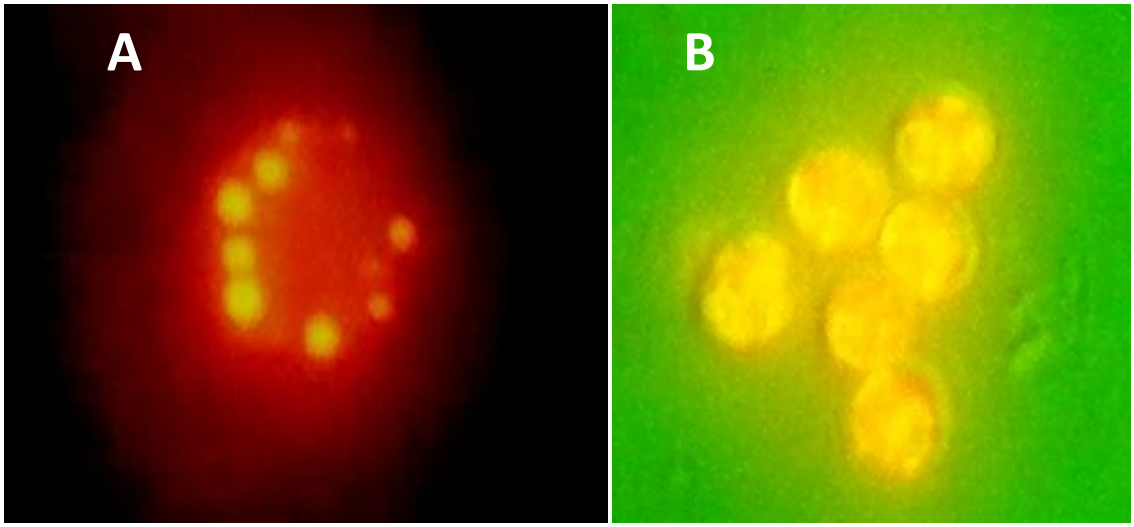


Figure 9: Fluorescent microscopy of cells stained with Nile red. A is a single cell grown in nitrogen rich media; yellow bodies are oil droplets; B shows a cluster of cells in media depleted of nitrogen. (Magnification 40X, excitation 485nm, emission 525nm).

It has been hypothesized that as nitrogen becomes limiting, algae will begin using chlorophyll as a nitrogen source to continue molecular development and division (Gouveia *et*

al., 2009). The rest of the chlorophyll then is likely converted by the organism into the fatty acids and lipids that accumulate in the cell (Li *et al.,* 2008).

1.4 Algal Candidate for Biofuel Production, *Botryococcus braunii*

Botryococcus braunii is a colonial green alga (Figure 10) known to produce long chain hydrocarbons that are secreted in its extracellular matrix. These algae are naturally found all over the world, including fresh or brackish waters, and in nearly any climate (Metzger and Largeau, 2005). Figure 10 displays an image of *B. braunii* which shows the algae growing in colonies.

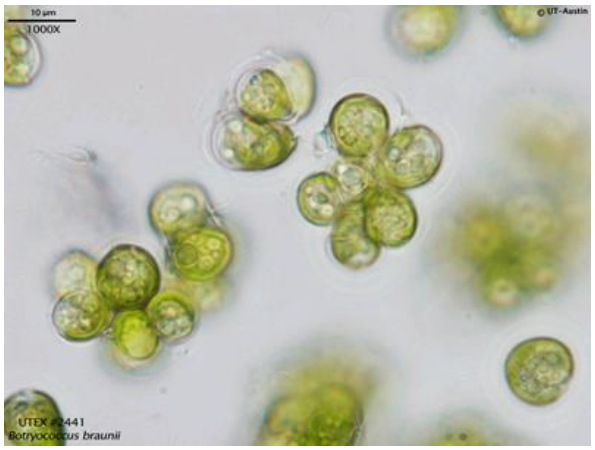


Figure 10: *B. braunii* growing in colonies (taken from UTEX, 2010).

B. braunii comes in three main races, identified by the hydrocarbon product they produce. Race A predominantly produces alkadienes and alkatrienes containing 26-31 carbons (Banerjee *et al*., 2002). Race B produces triterpenes of C_{30}-C_{37} and methylated squalenes of C_{31}-C_{34}, also known as botryococcenes (Metzger and Largeau, 2005). Race L produces lycopadiene, $C_{40}H_{78}$ (Banerjee *et al*., 2002). The hydrocarbon products from the different races of *B. braunii* are displayed in Figure 11. *B. braunii* produces these hydrocarbons as part of its colonial matrix. The matrix is made up of hydrocarbons within layers of cell walls left over after cellular division (Metzger and Largeau, 2005).

Investigations have proposed that the hydrocarbons and biomass of *B. braunii* significantly contributed to several oil shales and deposits. Indeed, evidence shows the presence of *B. braunii* specific hydrocarbons and cellular characteristics in various petroleum products around the world (Banerjee *et al*., 2002).

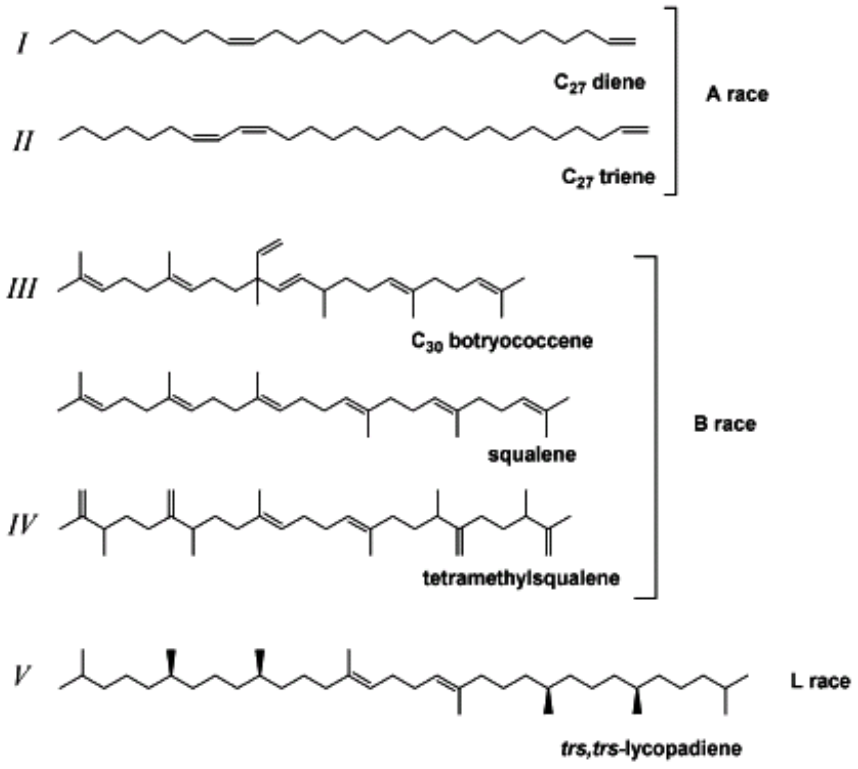


Figure 11: Comparison of the major hydrocarbon products of *Botryococcus braunii* strains A, B, and L (taken from Banerjee *et al*., 2002).

Hydrocarbons produced by *B. braunii* are of interest for biofuel production. They can be refined by conventional hydrocracking, a process currently in use at oil refineries today (Tran *et al*., 2010). Thus these hydrocarbons can easily be converted into fuels that can immediately be added to the market without any alteration to either the existing fuel infrastructure or vehicle design. Specifically, Race B of *B. braunii* is of most interest for the production of biofuels as its products are most easily converted to useable transportation fuels and may constitute 27-86% g/g dry weight of the cell (Banerjee *et al*., 2002).

Hydrocracking of *B. braunii* oils results in a 67% fraction of gasoline, 15% jet fuel, and 15% diesel fuel (Tran *et al*., 2010). The use of catalysts has the potential to adjust the fractional percentages, and increase the quality of the product. The ability to use existing refineries may help to decrease the amount of land area needed to make an economic impact on the US oil market, since companies may have the option to sell *B. braunii* products to refineries as crude oil instead of having their own refining process on site.

The main challenge to overcome before *B. braunii* can be exploited for oil production is the slow growth rate of the species. The growth rate has been increased, however, with

changes in the culture conditions. Most notably, when compared to growth with ambient air, the use of CO_2 enriched air decreased the doubling time from 6 to 1.7 days (Banerjee *et al*., 2002).

1.5 Algal Transformations

It would be useful to have a known transformation protocol that could be used to adjust the metabolic pathways to increase lipid yields, growth rates, and/or photosynthetic efficiency in order to create a more profitable strain of algae for biofuel production. A successful genetic transformation involves the incorporation of foreign DNA into the nuclear or chloroplast genome. The modified organism must be viable after the transformation, and the inserted gene must have the ability to be translated into a functional protein in the organism (Rosenberg *et al*., 2008). Successful nuclear and/or chloroplast transformations have been accomplished in several algal species (). The most frequent and successful methods of transformation involve either electroporation or microparticle bombardment, but species also have been transformed using glass bead agitation, silicon carbide whiskers, as well as *Agrobacterium tumefaciens* mediated genetic transfers (Coll, 2006; Rosenberg *et al*., 2008.). The most frequent and successful methods of transformation involve either electroporation or microparticle bombardment, but species also have been transformed using glass bead agitation, silicon carbide whiskers, as well as *Agrobacterium tumefaciens* mediated genetic transfers (Coll, 2006; Rosenberg *et al*., 2008).

While electroporation and glass bead agitation are both simple and inexpensive methods for transforming any organism, the drawback for use with algae is the requirement for cell wall deficient or cell wall-less organisms (Coll, 2006; Rosenburg *et al*., 2008). This obstacle was overcome in well studied species such as *Chlorella vulgaris* and *Chlamydomonas reinhartii* because cell wall deficient mutants and methods for protoplast formation are available (Coll, 2006). Transformation efficiencies of 2,770 transformants per million (0.28%) were obtained via the electroporation of cell wall-less *C. reinhardtii* (Coll, 2006).

Removal of the cell wall, and use of polyethylene glycol (PEG), a membrane fusion agent, improved the transformation efficiencies of glass bead agitation to 0.01% (Coll, 2006). The cell wall can normally be removed via enzymatic processes for well-studied plants and algae; however, the diversity of algal cell walls has kept transformations by these methods difficult to apply to every species of microalgae (Coll, 2006).

Microparticle bombardment uses high speed gold particles coated in DNA to penetrate the cell, and does not require the removal of the cell wall. However, very expensive instrumentation is needed, and such technology is not readily available to all labs and startup companies that may be interested in attempting algal transformations (Coll 2006). Also, the

maximum transformation efficiency observed so far with this method is only about 0.01%, about the same as for glass bead agitation (Coll, 2006).

Table 3: Species of algae that have previously been transformed (taken from Coll, 2006)

Class	Species[1]	Water	Ploidy[2]	Motile	Shape[3]	Size[4] (~ µm)	Cell-wall
Chloroficeae (green algae)	*Chlamydomonas reinhardtii*	Fresh	Haploid	Yes	Ovoid	10×5	Elastic thin
	Volvox carteri	Fresh	Haploid	Yes	Round	8	Elastic thin
	Chlorella ellipsoidea	Brackish	Diploid?	No	Ovoid	4	Rigid polysaccharides
	Chlorella saccharofila	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella vulgaris	Fresh	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella sorokiniana	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Chlorella kessleri	Brackish	Diploid?	No	Round	4	Rigid polysaccharides
	Dunaliella salina	Marine	Diploid	Yes	Ovoid	18×9	Elastic thin
	Haematococcus pluvialis	Fresh	Diploid	Yes	Round	25	Mucilaginous
Bacillariophyceae (diatoms)	*Phaeodactylum tricornutum*	Marine	Diploid	No	Long	16×3	Hard siliceous
	Navicula saprophila	Fresh	Diploid	Yes	Long	3×0.5	Hard siliceous
	Cyclotella cryptica	Fresh	Diploid	No	Round	5	Hard siliceous
	Thalassiosira weissflogii	Marine	Diploid	No	Round	3	Hard no siliceous
Dinophyceae (dinoflagellates)	*Amphidinium klebsii*	Marine	Haploid	Yes	Round	15	Rigid cellulose theca
	Symbiodinium microadriaticum	Marine	Haploid	Yes	Round	15	Rigid cellulose theca
Euglenophyceae	*Euglena gracilis*	Fresh	Haploid	Yes	Ovoid	50×20	Elastic thin
Rodophyceae (red algae)	*Porphyridium* sp.	Marine	Diploid	No	Round	18	Sulphated polysaccharides
	Cyanidioschyzon merolae	Hot water	Haploid	No	Ovoid	2	Polysaccharides

[1] Most of the species are unicellular, except *Volvox carteri,* which is pluricellular but with few cells. The names of the different *Chlorella* spp. are those given by the authors of the corresponding reports (according to the UTEX catalogue; however, some of these might be synonymous). *Cyanidioschyzon merolae* is an extremophile that can survive at pH < 1, 45ºC and 100% CO_2; it has one of the smallest genomes of all eukaryotic cells (16 Mbp). [2] Ploidy in some of the species is difficult to define because of the alternation between haploid and diploid stages. [3] Shapes have been classified as round, ovoid and long. [4] The size offered is an approximate average of the figures given by different authors.

Agitation of cells in a mixture containing PEG, plasmid DNA and silicon carbide whiskers was used to transform two dinoflagellates, *Amphidinium sp.* and *Symbiodinum microadruaticum* (Lohuis and Miller, 1998). While it was possible to transform the two species without removing the cell wall, only about 0.0025% of cells were transformed.

The process of obtaining even these efficiencies required several attempts at optimizing the procedure for electroporation and protoplast formation. Every step in the electroporation procedure can affect the ability of transformants to grow and multiply after DNA introduction. By altering the conditions for protoplast formation, electroporation, and post-transformation recovery, Tang *et al*. (1998) was able to achieve transformation efficiencies of 0.27% via electroporation of cell wall-less *C. reinhardtii,* a 1000 fold increase from the 0.0025 % previously achieved (Coll, 2006). Tang *et al*. (1998) were able to optimize every step in their procedure to

obtain the most transformants possible for *C. reinhardtii;* however, it is likely these conditions will not be universally effective for the electroporation of all algal species. Microalgae are a very diverse group of organisms with variations in cell wall chemistry, and it is likely that each species will require optimization of a transformation process.

For each separate species of algae the proper enzymes and growth conditions must be determined for the protoplasting step. The cell wall of the specific microalgae being studied will determine the enzymes used, but also the media that best facilitates protoplast survival and stability. Typical enzymes used for cell wall degradation include cellulose, pectinase, and macerozyme (macerase).

A. tumefaciens mediated transformation has also been successfully performed on two algal species, *C. reinhardtii* and *Haematococcus pluvalis,* without the removal of the cell wall (Kumar, 2004; Kathiresen *et al*., 2009). *A. tumefaciens* is the causative agent of crown gall disease in higher plants, inducing tumors in plants by inserting a piece of plasmid DNA, called the T-plasmid (Transfer plasmid) (Zupan *et al*., 2000). The ability of *A. tumefaciens* to insert DNA into complicated eukaryotic plant cells has been exploited for many years to transform plants for many different applications (Zupan *et al*., 2000). The process of transfection by *A. tumefaciens* is displayed in Figure 12.

A. tumefaciens mediated transformation of algae is still in its infancy, as prior work only showed feasibility of successful transformation and studied the effectiveness of the promoters and the presence of foreign genes after sexual reproduction (Kumar, 2004; Kathiresen *et al*., 2009). *A. tumefaciens* mediated transformation yielded a transformation efficiency for *C. reinhardtii* of 0.035% (Kumar *et al*, 2004), and 0.015% for *H. pluvalis* (Kathiresen *et al*., 2009). These transformation efficiencies are not very different from those using electroporation (Coll, 2006)

Preliminary attempts at over expressing genes in the lipid metabolism pathway have not resulted in an increase in lipid content of cells. The acetyl CoA carboxalase gene was over expressed in the diatom *Cyclotella cryptic,* but no increase in oil was observed (Rosenburg, 2008). On the other hand, attempts in silencing genes in the Light Harvesting Complex of *C. reinhardtii* resulted in a decrease of the effects of photo-inhibition (Rosenburg, 2008). Wang *et al*. (2009) was able to demonstrate that the use of a starchless mutant *cw15 sta6* (BAFJ5) of *C. renihardtii* resulted in a 15-fold increase in TAG production. These novel approaches are the first attempts at genetic modification outside of lipid biosynthesis. For the purpose of increasing TAG yields, the switch from starch to lipid production in BAFJ5 has also been the most successful.

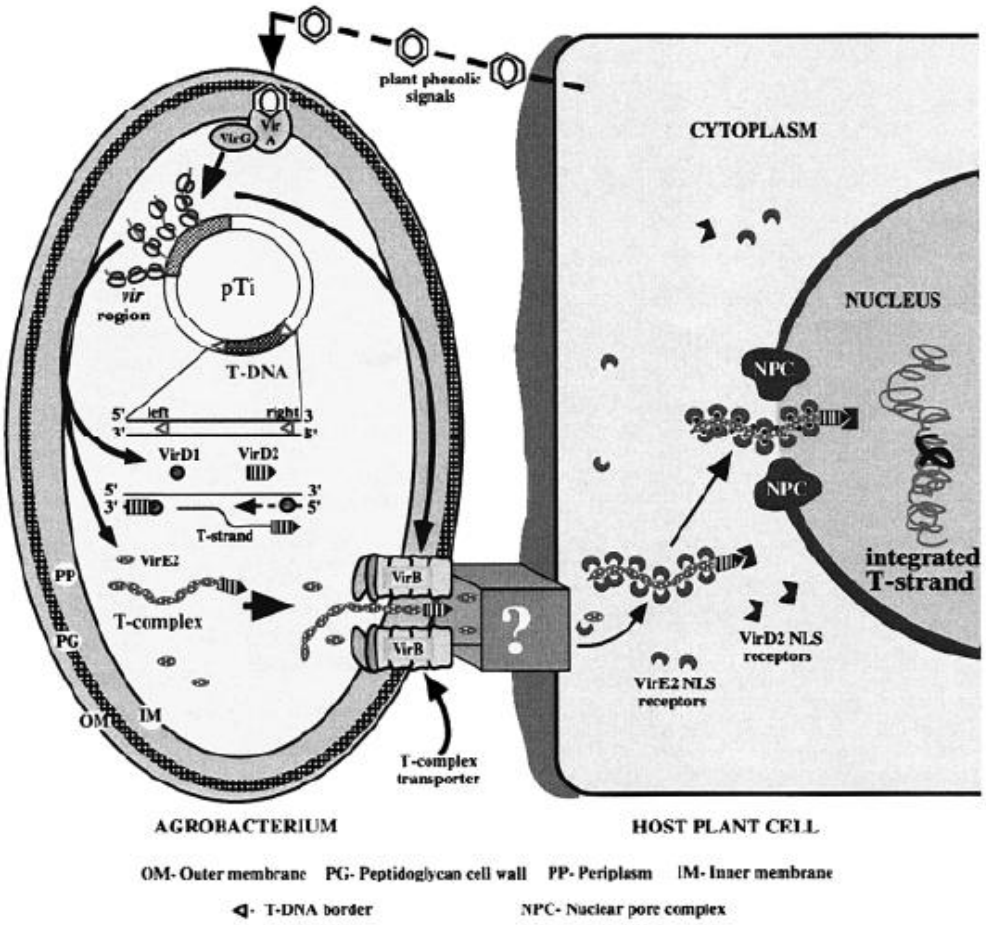


Figure 12: Transfection by *A. tumefaciens* (taken from Zupan *et al*, 2000).

Chapter 2: Hypothesis and Objectives

The goal of this project was to optimize the protocol developed by Carter (2010) in order to efficiently develop transformants. From this goal the following hypothesis was formed:

If it is possible to determine the concentration of protoplasts and their viability at each major step in the developed electroporation protocol, it will be possible to optimize the methodology to increase the ability to transform *Ettlia oleoabundans.*

From this hypothesis the following objectives were identified:

- Determine the optimal media to form protoplasts
- Determine if the presence of 0.4M D-sorbitol in all media and solutions results in increased survival and viability of protoplasts
- Determine the amount of protoplasts destroyed during the electroporation procedure

Chapter 3: Methods

3.1 Maintenance of Stock Cultures

Stock cultures of 30ml of *E. oleoabundans (UTEX 1185)* and *B. braunii* (Showa strain, UC Berkeley) were maintained in 125mL Erlenmeyer flask with Bold's Basal Media (BBM- see Appendix A) on a shaker bed at 25^{o}C and 100rpm to allow for proper aeration and to prevent settling of the algae. BBM was selected as growth media because it was shown during laboratory preparation to promote rapid growth of algal cultures.

Every two weeks, 5mL of each algal stock was subcultured and inoculated into 25mL of BBM. During every other round of subculturing, a contamination check was made. Samples of culture were streaked onto Petri dishes of BBM containing 20g/L of T.C. agar and placed under a light. Every 4 weeks samples were taken from stock cultures and streaked onto LB media containing 15g/L of T.C. agar in order to stimulate microbial growth, to determine if any there was any bacterial contamination.

3.2 Antibiotic Bioassay

The antibiotics augmenton and hygromycin were tested for their effects on the growth of *E. oleoabundans.* Both antibiotics were dissolved in diH_2O and filter sterilized through a 0.2µm Whatman Filter. Concentrations of 0, 222, 333, and 666mg/L were tested. To test the effects of augmenton, cultures without antibiotics were first grown to an OD540 of approximately 1.2. Once this OD was reached, augmenton was added to the samples at each of the above concentrations, keeping the total culture volume consistently at 15ml. The OD540 was measured over 4 days. The effects of hygromycin in liquid culture were similarly tested.

Hygromycin was also tested in solid agar media by inoculating plates with the different concentrations. A 500µl aliquot of *E. oleoabundans* from a seven day old culture was spread over the plate. Plates were placed under light at 25^{o}C and observed daily for the presence of colonies.

3.3 Genetic Constructs and Storage

Genetic Constructs for this experiment were developed by Carter (2010). His methods for building the different constructs, validation and their storage can be found in Section 3.3 of his MQP "Transformation of *Ettlia oleoabundans*, a Potential Biofuel Alga" and also in Appendix B. All constructs were revalidated in this project using the same methods.

3.4 Agrobacterium tumefaciens Stocks

Deactivated electrocompetent *Agrobacterium tumefaciens* LBA4404 cultures were stored in 20% glycerol at -80^{o}C. Overnight and control samples of LBA4404 needed for transformation and other uses were grown in LB media with 25g/l rifampicin for selection of the T-vector.

3.5 *A. tumefaciens* Transformation

Plasmid DNA was isolated from TOP 10 *Escherichia coli* with standard alkaline lysis protocol (Vanderbilt, 2011) and purification was measured using Nano Drop UV 260/280nm fluorescence. Overnight cultures of *A. tumefaciens* grown in LB media containing 25mg/L rifampicin were used as the host for electroporation. A 0.5ng aliquot of DNA was added to 25µl of *A. tumefaciens* culture, microfuged at 1000 x g for 20 seconds, and then allowed to incubate on ice for 40 minutes. A 25µl aliquot of cells was placed into a sterile electroporation cuvette. Electroporation parameters were: 4kΩ and 330µF. After electroporation samples were added to 1ml sterile LB and allowed to incubate for one hour. After one hour, 10µl and 100µl of samples in LB media were spread on LB agar containing both 25mg/l rifampicin and 50mg/l kanamycin. Colonies formed were counted to determine efficiency.

3.4.4 *A. tumefaciens* Confirmation

The presence of gene constructs was confirmed by restriction digest. Plasmid DNA was isolated from *A. tumefaciens* using the alkaline lysis protocol (Vanderbilt, 2011), and purity was again determined using Nano Drop. A 0.5ng aliquot of isolated DNA was mixed with 25µl TOP 10© *E. coli* and electroporated at 4kΩ and 330µF. Samples were then added to 1ml SOC media (Sigma) and placed in an incubator at 37^{o}C for 1hour. A 30µl aliquot was plated on LB agar with 50 mg/L kanamycin and grown overnight. Colonies were picked off the plate and grown overnight in liquid LB media containing 50mg/L kanamycin. Plasmid DNA used for the restriction digest was isolated from overnight cultures using the alkaline lysis protocol.

Samples were cut using EcoRV restriction enzyme. The enzyme blunt cuts on either side of the inserted gene. The digest was run on 1% agarose TAE gel with 1% Ethidium Bromide and visualized under UV light.

3.6 *A. tumefaciens* Mediated Transformation

E. oleoabundans and *B. braunii* were expected to be transformed, confirmed, and studied according to the protocol found in Appendix C with the following change: Augmenton was used to remove *A. tumefaciens* after co-cultivation.

3.7 Protoplast Formation and Algal Electroporation

The protoplasting methodology was modified from section *3.4 Formation of Protoplasts* and section *3.7 Transformation by Electroporation and Selection* from the methods in the MQP "Transformation of *Ettlia oleoabundans*, a Potential Biofuel Alga" by Carter (2010; Appendix B).

3.7.1 Confirmation of Protoplast

To optimize the process of protoplast formation in order to increase survival of protoplasts for electroporation and to enhance the chance of obtaining transformants, several conditions and media were tested for protoplast efficiency and growth throughout the electroporation process. The conditions tested are summarized as follows: Enzyme Solvent – BBM + sorbitol, H_2O + sorbitol, PBS + sorbitol; Electroporation Media – PBS + sorbitol, PBS - sorbitol; Recovery Media – BBM - sorbitol, BBM + sorbitol. Various combinations of these conditions were tested.

E. oleoabundans was grown until reaching mid log phase. Cells were subjected to cell wall degradation by 1/3 volume of a solution of 1% cellulose(w/v), 1% macerase(w/v), 1% pectinase (w/v) in 0.4M D-sorbitol in one of 3 solvents, water, BBM, or PBS, in 3ml test tubes. Digestion was allowed to proceed in the dark for 24 hours at 25^{o}C with gentile agitation on a slant rank on a shaker at 100 rpm. After 24 hours samples were pelleted in a microfuge at 100 X g for 10 minutes, washed with 0.4M D-sorbitol, and stained with 1 volume of 0.1% calcofluor dissolved in 50% NaOH ddH_2O for 1 hour. Samples where then placed in a microfuge at 100 X g and then washed twice in 0.4M D-sorbitol. Final samples were resuspended in 1/5 volume 0.4M D-sorbitol.

Samples were viewed using a confocal microscope to determine if the wall was completely degraded. A 5µL aliquot of each sample was transferred to a microscope slide with a cover slip. The edges of the cover slip were sealed using clear nail polish. The stain was excited at 365nm with an emission wavelength at 435nm. Images were taken using an Olympus Industrial Microscope and Lyca software.

3.7.2 Electroporation

Once protoplasts were formed, cells were placed in a microfuge at 100 X g, supernatant removed and the pellet re-suspended in Phosphate Buffered Saline, PBS (electroporation media). A 0.5ng aliquot of DNA was added to 25µl of re-suspended cells and placed on ice for an hour. The samples were then subjected to electroporation of 850V/cm at 10µF. After electroporation samples were incubated for an hour in 1ml BBM (recovery media) then spread onto BBM agar plates containing hygromycin for selection of any putative transformants.

3.8 Quantitation of Protoplast Formation

To test the efficiency of protoplast formation, a simple procedure, the "pop test", was adopted to test the osmotic rupturing of the cells without cell walls. After the 24 hour incubation period in the wall degrading enzymes, an aliquot of cells was re-suspended in either 0.4M D-sorbitol or ddH_2O. Cells lacking cell walls and suspended in the 0.4M D-sorbitol were expected to be protected from rupturing due to the osmotic protection of the sorbitol, however, the cells suspended in ddH_2O that lost their wall due to enzymatic degradation are expected to rupture due to the osmotic imbalance between the media and the cytosol of the cell. Based on this hypothesis the protoplast efficiency was determined by counting the number of intact cells in ddH_2O and comparing it to the number of cells suspended in 0.4M D-sorbitol.

3.9 Statistical Analysis

For all experiments run in triplicate, a T-test statistical analysis was performed. In some cases there has to date been only 1 experiment conducted; replications will be completed later.

Chapter 4: Results

4.1 Hygromycin Sensitivity

To select for a successful transformation, an antibiotic capable of inhibiting or preventing the growth of *E. oleoabundans* must first be determined. Once a proper selection medium is determined, the corresponding resistance gene can be used to more easily select for transformants by observing colonies that are capable of growing on media containing the antibiotic. It was determined that the growth of wild type *E. oleoabundans* is strongly inhibited by the antibiotic hygromycin (Figure 13). When log-phase cultures were used to inoculate liquid BBM media containing 5µg/ml-100µg/ml, growth was completely inhibited by hygromycin. In additional, agar-based solid BBM mediium containing hygromycin yielded no colony growth of *E. oleoabundans* at any of the tested concentrations, confirming hygromycin resistance as a proper selectable marker.

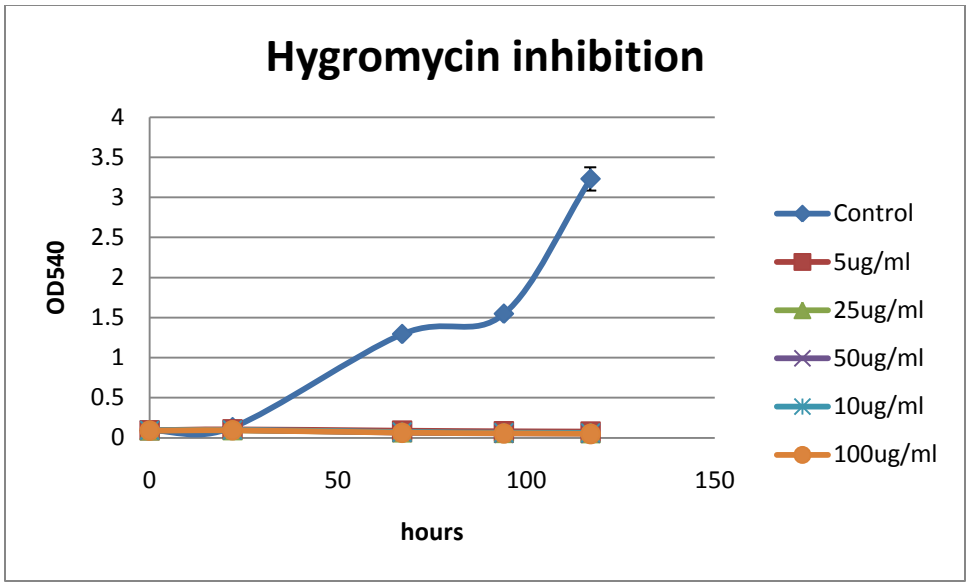


Figure 13: Growth inhibition of *E. oleoabundans* by hygromycin.

4.2 Genetic Construct Validation for the Transformation of *Ettlia oleoabundans* and *Agrobacterium Tumefaciens*

The genetic constructs used in an attempt to transform *Ettlia oleoabundans* and *Agrobacterium tumefaciens* were developed by Carter (2010). The backbone is the pCAMBIA 1300 vector (Figure 14). It was chosen because it has an origin of replication and the Kanamycin resistant gene for both *E. coli* and *A. tumefaciens.* These characteristics allowed for ease of replication in *E. coli* as well as for use in *A. tumefaciens* mediated genetic transfer. The construct also contained the resistance gene to hygromycin under control of the CaMV 35s promoter as a selectable marker. Carter (2010) also separately ligated the reporter genes, green fluorescent protein (GFP) and beta-glucuronidase (GUS), with the CaMV 35s promoter into the multiple cloning site of the pCAMBIA 1300 backbone. GFP was also ligated with the nos promoter sequence. The genetic constructs within samples were verified to insure the presence of the inserted genes by restriction digests and agarose gel electrophoresis (Figure 15).

As can be seen in Figure 15 the plasmid samples taken from *E.coli* (lanes 8, 9 and 10) clearly contain the pCAMBIA 1300 backbone as well as a copy of the inserted gene. However, *A. tumefaciens* samples were consistently not of quality to confirm the presence of one inserted gene. Lanes 2-7 are indicative of gels run prior to this one, showing more bands than should be present as well as, at best, weakly visible bands. Without being able to confirm that the DNA to be used to transform *E. oleoabundans* was present in *A. tumefaciens* samples, it was not possible or of interest to continue the protocol for *A. tumefaciens* genetic transfers (Appendix B).

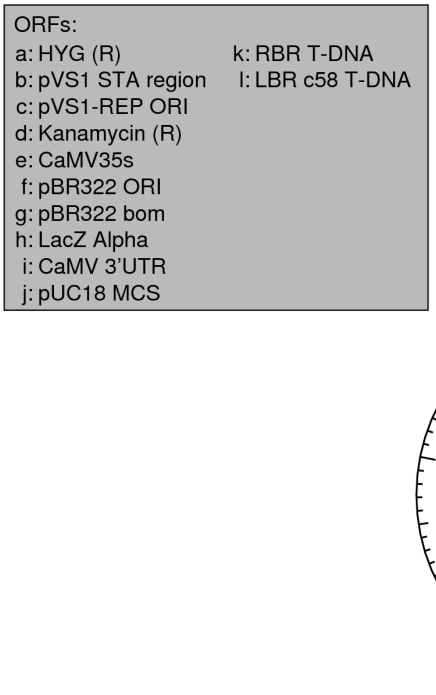
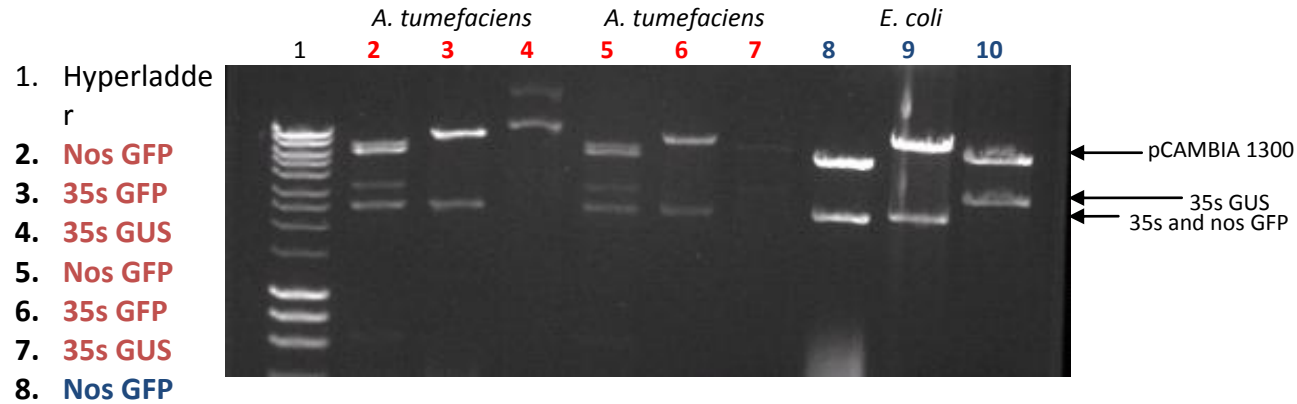


Figure 14: Restriction map for pCAMBIA 1300 PvuII (Taken from Carter, 2010)



1. Hyperladder
2. **Nos GFP**
3. **35s GFP**
4. **35s GUS**
5. **Nos GFP**
6. **35s GFP**
7. **35s GUS**
8. **Nos GFP**
9. **35s GFP**
10. **35s GUS**

Figure 15: Genetic constructs of *A. tumefaciens* and *E. coli*

4.3 Protoplast Formation

Past successful algal transformation methods involving electroporation required the removal of the cell wall either through the use of mutants (wall-less mutants) or enzymatic degradation of the wall. Based on previous work done by Carter (2010), a mixture of pectinase, cellulase, and macerase was known to be a promising combination of enzymes for removing the cell wall of *E. oleoabundans*. These three enzymes, respectively, degrade pectin, cellulose, and hemicelluloses and pectin. Calcofluor is commonly used to stain the cell wall of plants and was therefore used to visualize the formation of protoplasts. The stain readily binds to the cell

wall, but not the plasmalemma, and fluoresces at 435nm when excited with light at 365nm. Cells subjected to the enzymes were stained, and using a confocal microscope, were compared to stained control samples to determine if the cell wall was absent. Controls showed a clear bright blue "halo" around the cell auto-fluorescing chlorophyll (Figure 16 A), while no blue stain was visible in cells incubated in the enzyme mixture (Figure 16 B).

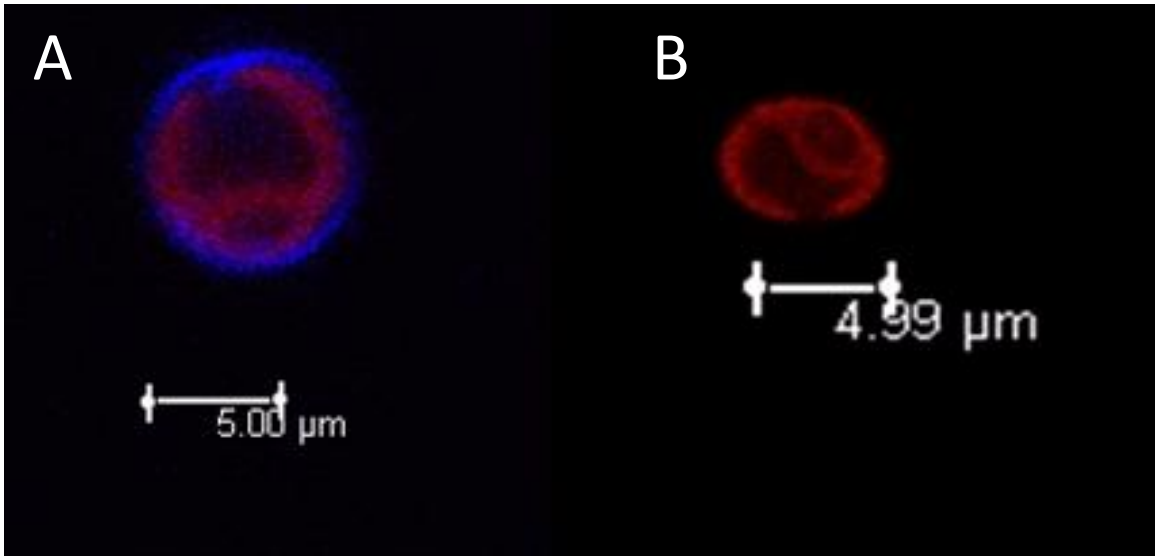


Figure 16: Confirmation of Protoplast Formation

Panel A is a picture of cell incubated in the absence of enzymes (positive control). Panel B is a picture of a protoplast cell after 24 hr incubation in the enzyme solution.

4.4 Procedural Optimization

Using the pop test and following the protocol developed by Carter (2010), approximately 25% of the cells were determined to be protoplasts; they were capable of osmotic lysis in ddH_2O after incubation in the enzyme solution (Figure 17). Carter (2010) had established what seemed to be a promising protocol for introducing exogenous DNA by electroporation by studying the effects that different voltages and capacitances had on the growth of *E. oleoabundans* (Appendix B). Although a high percentage of protoplasts were obtained through this procedure, no transformations were achieved. Also, in this study, control groups (cells subjected to electroporation in the absence of DNA and cells prepared for electroporation but not subjected to the procedure) grew poorly on semisolid 15% (w/v) agar BBM, often failing to produce any colonies if 0.4M d-sorbitol was not present in the semisolid media. From these results it was deemed important to reevaluate the procedure in order to produce a protocol with a higher likelihood of success.

To optimize protoplast formation, electroporation and the survival of cells, a number of variations in the protocol were systematically tested for protoplast efficiency and cell growth

(Table 4: Effects of 0.4M D-sorbitol on the growth of *E. oleoabundans* on semi-sold media). By using the pop test to measure the percent of cells that are protoplasts at each step of the protocol for each variation, as well as observing their subsequent growth on plates growth, it was possible to determine what step may be most likely (or unlikely) to produce a high number of protoplasts and, therefore, transformants. Samples were run so that the each variable's effect on growth could be compared relative to one another.

The four samples that grew the fastest and with fuller lawns on plates were those which contained 0.4 M D-sorbitol in the recovery medium (Table 4 A, C, E, and G), showing it had the most positive effect of any variable on the growth of samples post-electroporation. The fastest growing sample had its cell wall degraded in BBM, suggesting it may be the best medium for obtaining transformants (Table 4 G). However, significantly higher protoplast efficiencies were found in the samples degraded in ddH_2O compared to samples incubated in BBM, 25% and 17%, respectively (Figure 17), possibly indicating it as a better medium for protoplast formation.

Table 4: Effects of 0.4M D-sorbitol on the growth of *E. oleoabundans* on semi-sold media

(+ indicates the use of a given variable, - indicates the absence; growth was comparatively ranked, 1= fastest, 8=slowest)

	Enzyme Solvent		Electroporation Media		Recovery Media		Growth
	BBM+Sorbitol	H_2O+sorbitol	PBS+ sorbitol	PBS- sorbitol	BBM + sorbitol	BBM- Sorbitol	BBM Agar+ sorbitol
A	-	+	-	+	+	-	3
B	-	+	-	+	-	+	8
C	-	+	+	-	+	-	4
D	-	+	+	-	-	+	7
E	+	-	-	+	+	-	2
F	+	-	-	+	-	+	6
G	+	-	+	-	+	-	1
F	+	-	+	-	-	+	5

With the protocol augmented to optimize the growth of *E. oleoabundans,* transformations with the GFP and GUS constructs were attempted on protoplasts formed in both BBM and ddH_2O. No transformants were produced using either medium, suggesting other obstacles would need to be overcome.

The fragile nature of protoplasts makes them challenging to study and to maintain intact; therefore, it was important to know that the protoplasts that were initially formed survived to the step of electroporation. The resuspension and pipetting process needed to prepare samples for electroporation introduces sheer stress on the cells that would normally be

tolerated if a cell wall were present. However, these sheer forces placed on protoplasts can easily rupture the wall-less cells. To test if the steps between enzymatic digestion and electroporation reduced the number of protoplasts, protoplasting efficiencies were measured at the time of electroporation, and after resuspension in PBS+sorbitol and suspending the cells in the electroporation cuvette. The number of protoplasts in each sample decreased by 40-50% of the original amount made after being suspended in the cuvette (Figure 18). Indeed the number of intact protoplasts declined to 15% of the total cells in cultures degraded in H_2O, and dropped to under 10% in those degraded in BBM (Figure 17).

The loss of protoplasts during resuspension in PBS and preparation for electroporation suggested that digestion in PBS + sorbitol might reduce losses because one of the transfer steps could be eliminated. The hypothesis was that if the cells did not need to be pelleted and resuspended in PBS it may be possible to remove some of the sheer stress placed on the protoplasts leading to greater survival. Results of a recent (and the only) test showed that more protoplasts survived using this 2-step process than with the 3 step method (Figure 18). Furthermore, the protoplast efficiency for cells incubated in PBS + sorbitol and the cell wall degrading enzymes was higher than any other sample at both stages in the procedure (Figure 17). Since more protoplasts reach the stage of electroporation and cells grew at a comparable rate to the other samples with colonies appearing a week after inoculation, this variation in the procedure seemed to increase the chance of obtaining a transformant, however, to date none has been produced.

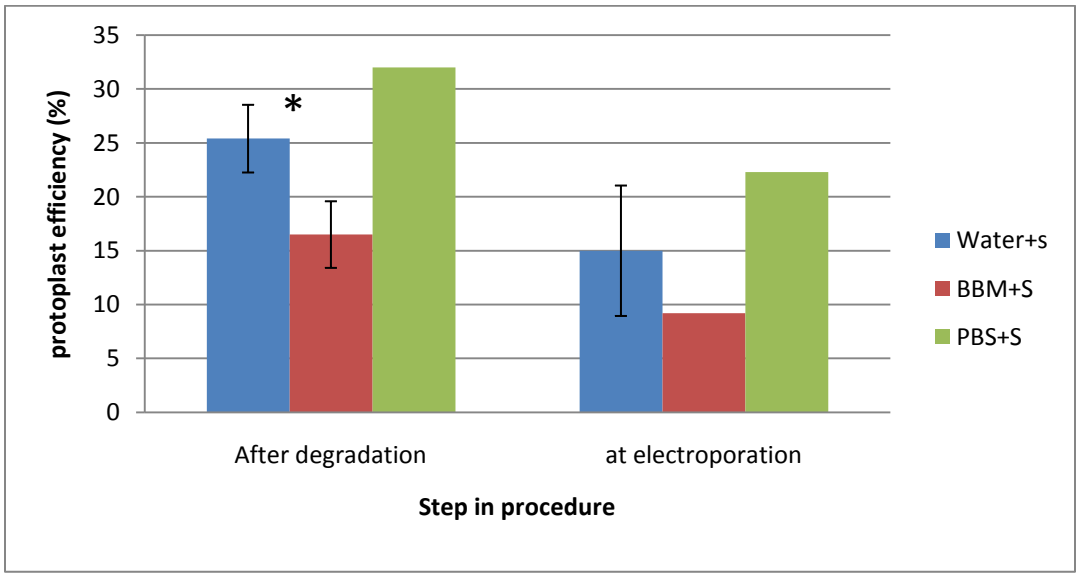


Figure 17: Protoplast efficiencies during electroporation protocol.

(Water and BBM samples were run in quadruplicate after degradation. Duplicate samples of water samples were tested at electroporation. One sample of PBS was tested for both steps.

One sample of BBM was tested at electroporation. Error bars represent standard deviation. *indicates significant difference p <0.01 where n ≥ 3)

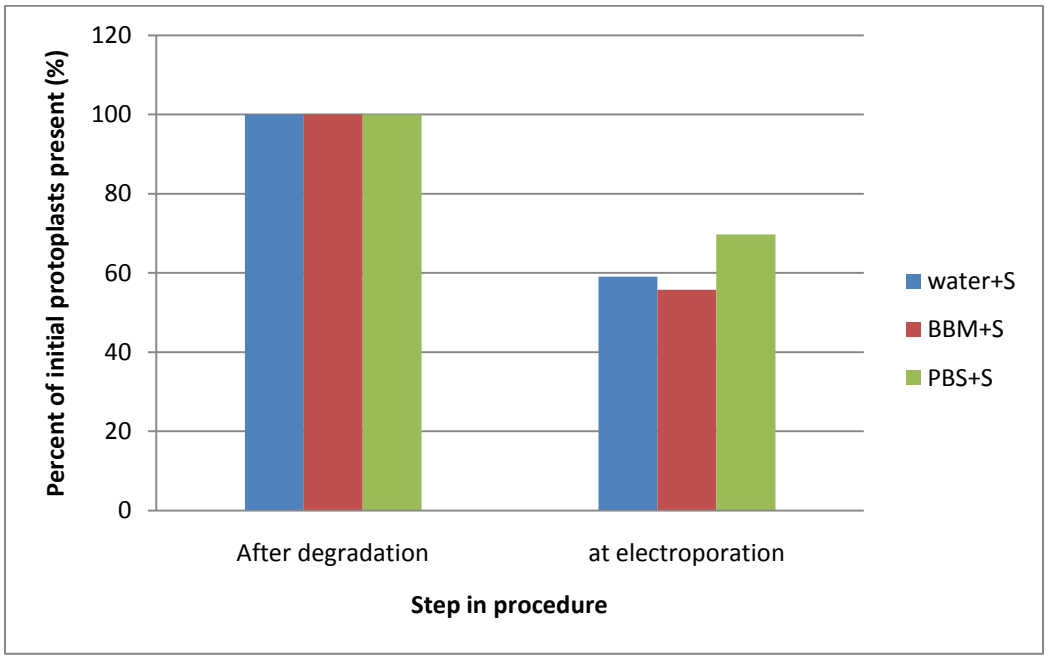


Figure 18: Percent of protoplasts surviving to electroporation.

Chapter 5: Discussion

Agrobacterium tumefaciens may be capable of transferring genetic constructs into *E. oleoabundans* without the removal of the cell wall. This method has the benefit of working with algal cells that are much less fragile than the protoplasts required for electroporation. However, the use of *A. tumefaciens* has not resulted in high transformation efficiencies and introduces another organism, which must be maintained and transformed. In this project it was difficult to validate that the plasmids being used to transform *E. oleoabundans* were truly present in *A. tumefaciens* samples. Plasmid DNA could not be visualized on agarose electrophoresis gels when isolated from *A. tumefaciens* cells, but needed to be replicated and isolated from *E. coli.* The added steps of transforming *A. tumefaciens* and *E. coli* proved to be difficult and unpredictable. *A. tumefaciens* mediated genetic transfer into *E. oleoabundans* was not attempted because of the inability to validate the genetic constructs within *A. tumefaciens.*

The electroporation of eukaryotic microalgae has required the removal of the cell wall in order to obtain high transformation efficiencies. Efficient methods of transforming *C. reinhardtii* have the advantage of using wall-less mutants (Tang *et al* 1998, Shimogawara *et al* 1998). The ability to grow mutant stains allows researchers to manipulate samples of cells that ideally consist entirely of protoplasts. With the optimization of conditions associated with the electroporation of *C. reinhardti,* efficiencies of $2x10^5$ transformants per µg DNA were achieved.

Variables optimized included osmolarity, temperature, concentration of exogenous DNA, voltage and capacitance.

For the less studied alga *E. oleoabundans,* wall-less mutants are not known, therefore, enzymatic degradation appeared to be a promising method for forming protoplasts. This procedure along with the conditions studied by those working with wall-less mutants also had to be optimized to obtain efficient transformation. Carter (2010) reported optimizing the voltage and capacitance used for electroporation, as well as developing a method to produce protoplasts at a stable osmolarity, which was confirmed using the fluorescent dye calcofluor.

In this project, conditions of the method developed by Carter (2010) were studied in an attempt to increase the survival and viability of protoplasts throughout the electroporation procedure. The media in which the cell wall digestion occurs affects the formation of protoplasts as well as the growth of the algae post-electroporation. Samples incubated in BBM grew faster on agar after electroporation, but developed approximately 5% fewer protoplasts. The use of 0.4M D-sorbitol in PBS and BBM in subsequent steps further increased protoplast survival and growth. Incorporation of sorbitol into the PBS solution used for electroporation had the greatest effect on growth for any of the liquid media used. The most notable effect D-sorbitol had on growth was when it was in the BBM agar used for plating cells after electroporation. Plates without D-sorbitol failed to grow colonies, while plates containing D-sorbitol showed robust growth within a week of inoculation.

E. oleoabundans protoplasts were most efficiently produced in PBS at 32% of the total cells. In comparison, 47.8% of *Chlamydomonas* sp. cells had their cell wall degraded by a mixture of cellulase and macerozyme (Liu *et al*., 2006). *E. oleoabundans* and *Chlamydomonas* cells are quite different; however it may be possible to achieve similar protoplast efficiencies through further optimization of the cell wall degradation procedure. Liu *et al*. (2006) adjusted the temperature and pH of the enzyme mixture in order to achieve this efficiency, suggesting more fine tuning of variables in this study may be helpful.

Considering the highest protoplast efficiency achieved at electroporation in PBS was 22.3%, it is safe to speculate that a transformation efficiency higher than 22.3% cannot be achieved. In optimizing electroporation for *E. oleoabundans* Carter (2010) found parameters that were effective at reducing the population of cells subjected to the pulse. The optimal electroporation parameter should reduce the viable population of a sample by 50%. Since electroporation has little effect on *E. oleoabundans* cells with a cell wall, then it is possible that up to 11.15% of cells will be capable of a transformation event due to the loss of 50% of protoplasted cells with negligible loss of walled cells.

When electroporation was optimized for the wall-less mutant of *C. reinhardtii,* transformation efficiencies of 0.27% were achieved (Tang *et al* 1998). If this efficiency was to be considered the optimum transformation efficiency that can be achieved with *E. oleoabundas,* one could speculate that the highest transformation efficiency achievable by this species would be 0.03% using the current method. Tang *et al*. (1998) were able achieve an efficiency of 0.27% working with 100% protoplast efficiencies in cultures of wall-less mutants. The ability to at best have 11.15% of cells capable of transformation coupled with low transformation efficiencies makes the probability of achieving an *E. oleoabundans* transformation by electroporation very low. The theoretical transformation efficiency of 0.03% is however slightly higher than any other method previously used to transform other species of algae (*A. tumefaciens*, glass bead agitation, microparticle bombardment, etc.) (Coll, 2006).

The method used to form and electroporate algal protoplasts is mainly based on the transformation methods commonly used for higher plants. Cell wall degradation and electroporation has been developed and used to transform the cells of higher plants for several decades and, in comparison, has been relatively well studied. As example, carrot cells, a staple in plant cell research, can yield 25-30% transformation of its protoplasts (Bower and Birch, 1990). Carrot cell protoplasts were produced using cellulase, macerozyme, and pectinase. The protoplasts were isolated from other cells using an isolation solution containing 0.37M glucose, 1.5mM CaCl, and 0.05% morpholinoethanesulphonic acid (Bower and Birch, 1990). The isolation of plant protoplasts, although not likely, provides the potential for nearly 100% of cells subjected to electroporation to be transformed. While algal electroporation methods are similar to those of higher plant cells, the efficiency of transformation for carrot cells is 100 fold higher than what has been achieved, so far, for algal cells (Bower and Birch, 1990).

Chapter 6: Conclusion and Future Work

The use of 0.4M D-sorbitol in all media used increased the survival of protoplasts, and the rate of growth after plating. Additionally, the enzymatic removal of *E. oleoabundans* cell wall was the most efficient when performed in PBS. This medium also allowed the cells to be directly electroporated without the need for resuspension, thereby avoiding steps that may rupture protoplasts.

While a transformation was not achieved in the scope of this project, the optimization of the procedure will ideally be the groundwork for others to use in future attempts to transform *E. oleoabundans* in order to develop it as a more feasible biodiesel feedstock. Additionally, the ability to form and maintain protoplasts could lead to the development of other transformation methods if electroporation does not efficiently produce transformants. With the cell wall removed liposome mediated genetic transfer as well as the use of PEG could

be explored as alternative methods of transformation as they function to disrupt the cell membrane in order to deliver DNA.

Future work can now focus on the genetic manipulation of *E oleoabundans* now that the protocol previously developed has been optimized for protoplast survival. The introduction and expression of exogenous DNA will still be difficult to perform on *E.oleoabundans* even with the consistent development and maintenance of protoplasts. Since nothing is really known about the genome of this algal species, it still may be difficult to obtain transformants that express the inserted genes. Different promoters may need to be explored as well as the use of introns and other types of enhancers perhaps specific to this algal species in order to efficiently produce transformants with the desired inherited traits (Coll, 2006). The constructs developed by Carter (2010) used in initial unsuccessful transformation attempts may still be useful, however, for transforming *E. oleoabundans.* A better understanding of the organism will likely also be needed before a modified line could become industrially viable. The methods developed in this project have helped to further develop that understanding.

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Appendix A

Bold's Basal Media (BBM)

To prepare the final medium, begin with 700-800mL of dH_2O and add 10 mL of the first six stock solutions. Add 1 mL each of the alkaline EDTA, acidified iron, boron and trace metals solutions. Autoclave. The final pH should be 6.4.

Component	400 mL Stock Solution	1 Liter Stock Solution	add quantity below per liter of medium	Molar Concentration in Final Medium
Major Stock				
$NaNO_3$	10 g L^{-1} dH_2O	25.00 g L^{-1} dH_2O	10 mL	2.94 x 10^{-3} M
$CaCl_2 \cdot 2H_2O$	1 g L^{-1} dH_2O	2.50 g L^{-1} dH_2O	10 mL	1.70 x 10^{-4} M
$MgSO_4 \cdot 7H_2O$	3 g L^{-1} dH_2O	7.50 g L^{-1} dH_2O	10 mL	3.04 x 10^{-4} M
K_2HPO_4	3 g L^{-1} dH_2O	7.50 g L^{-1} dH_2O	10 mL	4.31 x 10^{-4} M
KH_2PO_4	7 g L^{-1} dH_2O	17.50 g L^{-1} dH_2O	10 mL	1.29 x 10^{-3} M
NaCl	1 g L^{-1} dH_2O	2.50 g L^{-1} dH_2O	10 mL	4.28 x 10^{-4} M
Alkaline EDTA Stock Solution			add 1 mLof this solution per liter of medium	
EDTA anhydrous		50 g L^{-1} dH_{2O}		4.28 x 10^{-4} M
KOH		31 g L^{-1} dH_2O		1.38 x 10^{-3} M
Acidified Iron Stock Solution			add 1 mL of this solution per liter of medium	
$FeSO_4 \cdot 7H_2O$		4.98 g L^{-1} dH_2O		4.48 x 10^{-5} M
H_2SO_4		1.0 mL		
Boron Stock Solution			add 1 mL of this solution per liter of medium	
H_3BO_3		11.42 g L^{-1} dH_2O		4.62 x 10^{-4} M
Trace Metal Stock Solution			add 1 mL of this solution per liter of medium	
$ZnSO_4 \cdot 7H_2O$		8.82 g L^{-1} dH_2O		7.67 x 10^{-5} M
$MnCl_2 \cdot 4H_2O$		1.44 g L^{-1} dH_2O		1.82 x 10^{-5} M
MoO_3		0.71 g L^{-1} dH_2O		1.23 x 10^{-5} M
$CuSO_4 \cdot 5H_2O$		1.57 g L^{-1} dH_2O		1.57 x 10^{-5} M
$Co(NO_3)_2 \cdot 6H_20$		0.49 g L^{-1} dH_2O		4.21 x 10^{-6} M

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Appendix B

Selected from "Transformation of Ettlia oleoabundans, a Potential Biofuel Alga" by Brant Carter

3.3 Transformation vector preparation

The 35s-GFP, and 35s-GUS cassettes from pGreen (Hellens *et al*., 2000) plasmids were ligated into the multiple cloning site of the pCAMBIA 1300 plasmid (a gift from Dr. Argelia Lorence, Arkansas Biosciences Institute, Jonesboro, AR). DHSα cells in -80 °C cryostasis containing either pGreen 35s-GFP or 35s-GUS cassettes were thawed in LB media (Bertani, 2004; Appendix: Formulations) and cultured at 37 °C overnight. The plasmids were extracted from DHSα cells using the standard alkaline lysis protocol and then ethanol precipitated (see Appendix: Protocol Details).

Presence of the plasmids was verified by loading 1 µL of each sample onto a 1% agarose electrophoretic gel run for 60 min at 90 V. Each cassette was spliced out of its pGreen backbone with EcoRV (blunt cut) restriction enzyme (New England Biolabs, Inc., cat# R0195S) in 30 µL digest volumes (1 µL of 20 units/µL EcoRV, 3 µL DNA sample, 3 µL NEBuffer 3, 4 µL BSA, and 28 µL reagent grade diH2O) incubated for 90 min at 37 °C and then isolated by a 1% agarose electrophoretic gel extraction followed by phenol chloroform extraction and ethanol precipitation. GFP and GUS restriction maps are shown in Figure 4.

The pCAMBIA 1300 plasmid was digested with PvuII (blunt cut) restriction enzyme (New England Biolabs, Inc., cat# R0151S) in 30 µL digest volumes (1 µL of 5 units/µL PvuII, 4 µL DNA sample, 3 µL NEBuffer 3, 4 µL BSA, and 27 µL reagent grade di H2O) incubated for 90 min at 37 °C, dephosphorylated with shrimp alkaline phosphatase (SAP) (1 µL of 1 unit/µL SAP and 10x SAP Buffer), incubated for 15 min at 37 °C, heat shocked at 65 °C for 15 minutes, and then isolated by a 1% agarose electrophoretic gel extraction followed by phenol chloroform extraction and ethanol precipitation. See Figure 5 for the restriction map of the pCAMBIA 1300 plasmid.

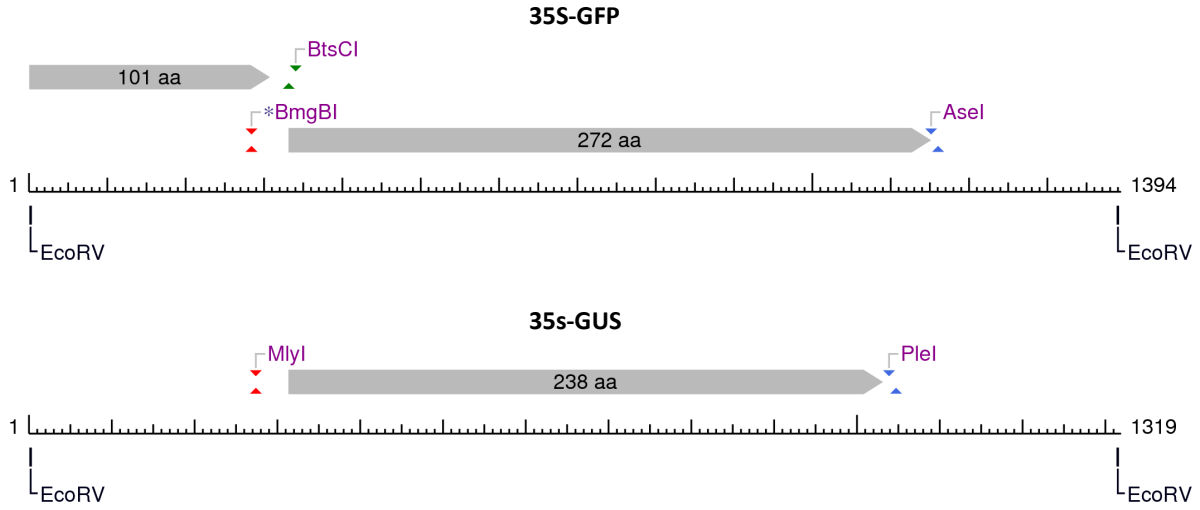

Figure 4: Restriction maps for 35s-GFP & 35s-GUS EcoRV. The pGreen backbone is not included; map was generated using NEBCutter 2.0 (Vincze *et al*., 2003)

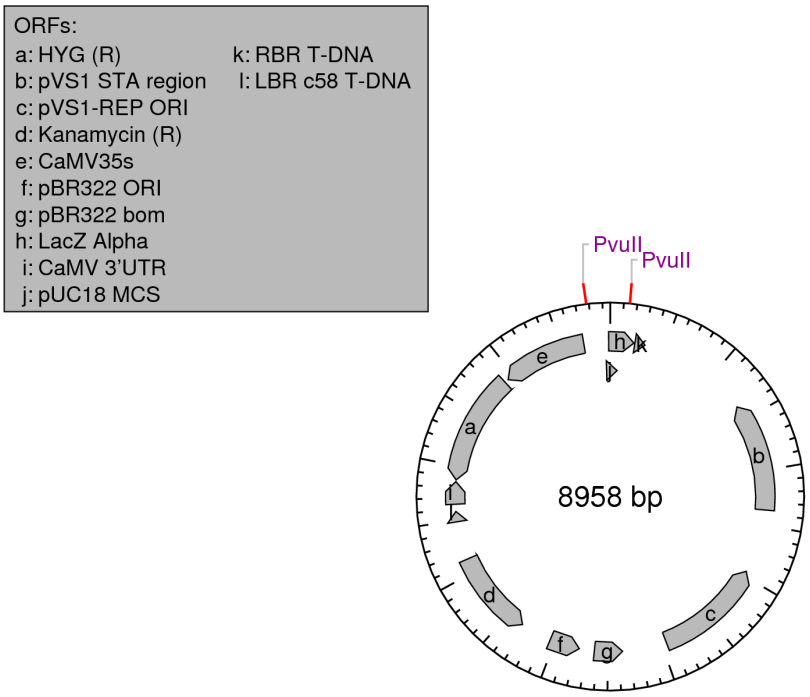

Figure 5: Restriction map for pCAMBIA 1300 PvuII . The pGreen backbone is not included; map was generated using NEBCutter 2.0 (Vincze *et al*., 2003)

The 35s-GFP and 35s-GUS inserts were ligated into open, dephosphorylated pCAMBIA 1300 backbones at an approximate 3:1 molar ratio in 20 µL working volumes with T4 DNA ligase (1 µL T4 DNA Ligase, 10x T4 DNA Ligase Buffer, and reagent grade DI H2O), and incubated overnight at 15°C. Ligands were transformed into heat competent *E. coli* and cultured in LB + Kanamycin (kanamycin resistance is conveyed by pCAMBIA 1300) overnight at 37° C and subsequently streaked onto LB Agar (1.5% w/v) + kanamycin plates for transformant selection. Surviving colonies were then picked and cultured again in LB + 100 µg/mL kanamycin overnight at 37 °C. The constructs were isolated from the cells by standard alkaline lysis protocol (Appendix: Protocols) and ethanol extraction (70% ethanol extraction followed by 100% ethanol extraction). Because the kanamycin resistance gene is part of the pCAMBIA 1300 backbone, selection with kanamycin does not ensure proper ligation of the inserts into the backbone. Therefore, construct digests with EcoRV were run on 1% agarose electrophoretic gels for 60 min at 90 V alongside a pCAMBIA 1300 PvuII digest, and 35s-GFP and 35s-GUS EcoRV digests to verify that proper constructs were present. Once verified, cells were prepared in glycerol solutions and put into cryogenic stasis with liquid nitrogen and stored at -80 °C.

3.5 Protoplast formation

Ettlia oleoabundans cells in log phase growth were subjected to cell wall digestion with 1% cellulase (w/v), 1% macerase (w/v), and 1% pectinase (w/v) in 0.4M D-sorbitol (Sigma Aldrich, St. Louis MO: S1876) for 24 hours at room temperature under gentle agitation. Following digestion, cell samples were centrifuged at 100xg for 10 minutes, washed with 0.4M D-sorbitol, and were stained with 1 volume of 0.1% calcofluor (fluorescent brightener 28; excitation 365nm; emission 435nm; Sigma Aldrich, St. Louis MO: F3543) for 1 hour. After staining, cells were centrifuged at 100 x g and washed twice in 0.4M D-sorbitol, and assayed for successful protoplast formation under UV fluorescent microscopy.

3.7 Transformation by electroporation and selection

Culture samples were taken in log phase growth, concentrated by centrifugation at 4,000 x g for 10 minutes to a factor of about 5 and electroporated with 600, 800, and 1000 V/cm at 10 µF with 2.5µg/mL DNA. Light/dark synchronized samples were taken from the synchronized culture at the transition from light to dark (12th hour in 24 hour cycle), 30 min after the transition, and 1 hour after the transition; these were all times where zoospore concentration appeared to be highest. Nitrate starved synchronized samples were taken at first sign of reproduction. Both types of synchronized cultures were then electroporated as previously described. Electroporated samples and one non-electroporated sample (control) were then cultured for approximately 4 days on BBM plates (75% of sample on plates with 20 µg/mL hygromycin; 25% of sample volume on control plates). Cultures were deemed to be successfully transformed if they showed hygromycin resistance conveyed by the hygromycin resistance gene (HYG) in the pCAMBIA 1300 backbone of each construct. Surviving colonies were assayed for GUS and GFP (excitation 395 nm; emission 509 nm) activity.

Appendix C

Genetic Transformation Protocols

From: Methods in Molecular Biology, vol. 344: Agrobacterium Protocols, 2/e, volume 2. Kumar *et al*., 2004. Pages 731-738

Green Alga (Chlamydomonas reinhardtii) Manchikatla V. Rajam and S. Vinod Kumar

2.3. Detection of GUS activity

1. X-gluc (5-bromo-4-chloro-3-indolyl-glucuronide) (Sigma).

2. GUS histochemical assay buffer: 10 mg X-gluc dissolved in 2 mL dimethyl for-mamide, 2 mL 5 mM potassium ferricyanide, 2 mL 5 mM potassium ferrocyanide. Make volume to 20 mL using 0.1M sodium phosphate buffer.

3. Incubator or dry-bath at 37°C.

4. Light microscope.

5. Protein extraction buffer: 50 mM Na2HPO4, pH 7.0, 10 mM β-mecaptoethanol, and 10 mM ethylene-diamine tetraacetic acid (EDTA) supplemented with 2 mM phenylmethylsulphonyl fluoride (PMSF).

6. MUG (4-methyl umbelliferyl-β-D-glucuronide) (Sigma).

7. GUS fluorimetric assay buffer: Extraction buffer containing 1 mM MUG.

8. Stop buffer: 0.2 µM Sodium carbonate.

9. 1 µM 4-methyl umbelliferone (4-MU): Dissolve in stop buffer.

10. Spectrofluorimeter.

11. Others: Standard reagents and materials for protein quantification according to standard procedure (15).

2.4. GFP Detection

1. Phase-contrast microscope (Nikon Eclipse TE 300 microscope with an excitation filter of 450–490 and a barrier filter at 520 nm).

2. Confocal laser scanning (Radiance 2100, Bio-Rad) using a Nikon microscope (objective Plan Apo 60X/1.4 oil, Nikon, Japan).

2.5. Isolation of Genomic DNA

1. DNA isolation buffer: 2% cetyl trimethyl ammonium bromide (CTAB), 1.4 MNaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.2% β-mercaptoethanol.

2. Chloroform:isoamyl alcohol (24:1 v/v).

3. Isopropyl alcohol.

4. Ethanol 70% (v/v).

2.6. Analysis of T-DNA Integration and Transgene Expression

1. PCR Buffer: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 100 μM deoxynucleotide triphosphate (dNTP) mix.

2. Taq-DNA polymerase (MBI, Fermentas).

3. PCR primers: 5′ -AGCTGCGCCGATGGTTTCTACAA-3′ (Forward primer) and 5′ -ATCGCCTCGCTCCAGTCAATG-3′ (Reverse primer) to amplify 0.5 kb fragment of hpt gene.

4. RNeasy plant kit (Qiagen).

5. Protein extraction buffer: 50 mM sodium phosfate buffer, pH 7.0, 10 mM Na2EDTA, 2 mM β-mercaptoethanol, and 2 mM PMSF.

6. Rabbit anti GUS polyclonal antibodies.

7. Goat anti-rabbit IgG—alkaline phosphatase conjugate (Sigma).

8. Others: Materials for Southern and Northern hybridization analysis according to standard procedures (16).

2.7. Mating and Genetic Analysis

1. C. reinhardtii strain CC-125 (mt+).

2. Gametogenesis media: Low sulfur medium (L-medium),

1/5 strength Nitrogen-free minimal medium (M-N/5 medium) (see Table 2 for the composition of the media).

3. Maturation medium: TAP plus 4% agar.

4. Zygospore germination medium: TAP plus 2% agar.

5. TAP agar plus 10 mg/L hygromycin.

6. Microspatula, blunt glass needles and mouth controlled pipet.

7. Stereo dissection microscope.

3. Methods

3.1. Culture and Maintenance of Chlamydomonas Unless otherwise mentioned Chlamydomonas cultures are grown in liquid TAP medium in an incubator shaker under continuous illumination using cool. fluorescent light with 60 µmol/m2/s at 100 rpm and 23°C. Solid cultures are always maintained on TAP-agar medium. All transformation experiments are initiated from a single colony-derived culture.

3.2. Transformation

1. Inoculate a single colony of Chlamydomonas into TAP medium and grow to log phase under illumination. Green Alga (Chlamydomonas reinhardtii) 4252. Plate about 107 cells from the log phase culture on to the solid TAP medium with or without 100 µMAS in 90-mm Petri plates and incubate in light for 2 d to allow a lawn of cell to be formed (see Note 6).

3. Raise an overnight Agrobacterium culture (A600 = 0.5) in liquid YEM medium containing appropriate antibiotics (in the present case 10 mg/L rifampicin, 50 mg/L streptomycin, and 50 mg/L kanamycin), pellet and resuspend in liquid TAP medium supplemented with or without 100 µM AS (see Note 7).

4. Plate 200 µL of the bacterial suspension on to the thin layer of Chlamydomonas culture growing on agar plates. Incubate the plates for 2 d at 23°C (co-cultivation)

5. Following 2 d of co-cultivation, harvest the cells, wash twice with liquid TAP medium containing 500 mg/L cefotaxime by re-suspending with mild vortexing and centrifugation at 100g for 2 min in a microcentrifuge, re-suspend in liquid TAP and plated 2–3 × 106 cells on to solid TAP plates containing 10 mg/L hygromycin + 500 mg/L cefotaxime.

6. Transformed colonies appear in 1 wk (see Note 10); maintain independent colonies on the selection medium with intermittent sub-cultures.

7. Utilize the transformed colonies maintained in liquid TAP medium under non-selective conditions for molecular analysis (see Note 11).

3.3. Detection of GUS Activity

1. For the analysis of gus reporter gene expression (17) transfer 1 mL of the culture to microcentrifuge tube, pellet the cells by centrifugation at 1500g for 5 min and re-suspend in 500 µL of GUS histochemical assay buffer.

2. Incubate cells in the assay buffer at 37°C overnight.

3. Remove the assay buffer by centrifugation, rinse the cells in 70% ethanol, and store in 40% glycerol until used.

4. Observe the cells under light microscope to visualize the GUS expression (see Fig. 2A).

5. For quantitative detection of the GUS activity, pellet 1 mL of culture in a micro- centrifuge at 1500g for 5 min at 4°C and homogenize the cells by sonication in the protein extraction buffer.

6. Centrifuge the homogenate at 13000g for 15 min at 4°C. Collect the supernatant and use for enzyme assay after quantifying proteins according to standard procedures (15).

7. Perform GUS assay by adding 100 µL of the extract to 500 µL of pre-warmed assay buffer and incubate at 37°C. Remove 100 µL aliquots at regular intervals and mix with 900 µL stop buffer.

8. Calibrate the fluorimeter or make a calibration curve using triplicate 0 to 100 nM 4-MU diluted in stop buffer as for the reaction samples (with excitation at 365 nm and emission at 455 nm) followed by reading the reaction sample fluorescence.

9. Extrapolate the enzyme activity from the standard curve of 4-MU and express as pmoles 4-MU/min/mg protein.

3.4. GFP Detection

1. For detection of green fluorescent protein (GFP) by phase-contrast microscopy, observe the cells mounted on a microscopic slide with a Nikon Eclipse TE 300 microscope with an excitation filter of 450 to 490 and a barrier filter at 520 nm.

2. For confocal microscopy, perform confocal laser scanning (Radiance 2100, Bio-Rad) using a Nikon microscope (objective Plan Apo 60X/1.4 oil, Nikon, Japan). Detect GFP fluorescence with an excitation of 488 nm (argon laser) and high quality band-pass emission filter HQ515/30, centered on 515 nm with 30-nm bandwidth.

3. Detect chlorophyll auto fluorescence through 637-nm red diode. Process the images in Photoshop

3.5. Isolation of Genomic DNA

1. To isolate genomic DNA from Chlamydomonas cells using CTAB buffer (18) pellet the cultures raised in 100 mL TAP medium to log-phase and re-suspend the cells in pre-heated isolation buffer.

2. Incubate the tubes at 65°C for 1 h with gentle mixing in between.

3. Extract the lysate with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by gentle mixing and centrifugation at 12,000g for 15 min at room temperature.

3. Separate the upper aqueous layer by gentle pipetting into a fresh tube, extract with equal volume of chloroform:isoamyl alcohol and centrifuge as described above.

4. Separate the aqueous layer and mix with two-thirds volume of iso-propyl alcohol in a fresh tube to precipitate the DNA. Mix the contents gently by inverting the tube several times, and centrifuge at 12,000g for 15 min at room temperature.

5. Wash the DNA pellet obtained with 70% ethanol, air dry, and dissolve in 100 µL of sterile distilled water.

6. Store DNA samples at −20°C until used for polymerase chain reaction (PCR) or Southern analysis.

3.6. Analysis of T-DNA Integration and Transgene Expression

1. Analyse the transformants by PCR with primers specific to hpt. Set up the PCR reaction mixture with 100 ng of DNA from untransformed control as well as putative transgenics with 100 nM of the transgene specific primers in 25 µL of PCR buffer containing 0.5 U of Taq DNA polymerase.

2. For Southern analysis, restrict 10 µg of genomic DNA with EcoRI and perform hybridization using 32P-labelled hpt gene fragment as a probe using standard procedures

3. Isolate total RNA from the transformed as well as wild-type cultures using the RNeasy Plant kit (Qiagen) according to manufacturers instructions

4. Use 30 µg of RNA from the transformed lines and the untransformed control for Northern blot hybridization using 32P labeled hpt gene fragment as a probe according standard procedures (16).

5. Separate soluble proteins (30 µg) extracted from the independently transformed colonies and untransformed cells of Chlamydomonas in the extraction buffer by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

4. After the separation, transfer the proteins on to PVDF membranes by electroblotting. Block the membranes with 3% bovine serum albumin (BSA) for 1 h and incubate with rabbit anti-β-glucuronidase polyclonal

antibodies for 1 h and detect by goat anti-rabbit IgG-alkaline phosfatase conjugate (Sigma) using standard procedures

3.7. Mating and Genetic Analysis

1. Prepare cells of both mating types for gametogenesis by growing separately on solid low-sulfur medium (L-medium) for 3 d (see Note 14).

2. Harvest the nutrient starved cells from step 1 and resuspended in one-fifth strength nitrogen-free minimal medium (M-N/5) at a density of 2 × 106 cells/mL and incubate with shaking under 18-h light to allow gametogenesis to occur.

3. Harvest gametes by centrifugation at 1500g for 5 min and resuspend in fresh M-N/5 liquid medium at a concentration of 0.5 × 108 cells/mL.

4. Mix equal number of both the gametes and allow mating for 1 h. A sample mating mixture can be observed under light microscope to monitor mating. Once mating is complete, plate the mixture onto maturation medium and incubate the plates under light for 24 h followed by incubation in the dark for 6 d to allow for maturation.

5. Remove the unmated gametes from the maturation plate by gently scrapping with a sterile razor. The zygospores will now be visible clinging on to the agar.

6. Collect the zygospores from the maturation plates using a microspatula or a mouth-controlled pipet under a dissection microscope; place on the germination medium and allow germinating for 1 d under light.

7. The zygospores undergo meiotic division and would be ready for the release of the tetrads in a day. Separate the tetrad products using blunt glass needle and allow producing independent colonies.

8. Colonies appear in 7 to 10 d. When colonies form, inoculate them into liquid TAP medium and allow to grow to a stationary culture.

9. Spot 5-µL aliquots of the stationary phase cultures on to TAP agar medium, with and without hygromycin, for screening of segregation of hygromycin resistance phenotype.

10. Use the tetrad products for PCR analysis to check whether the hygromycin resistance phenotype and the hpt transgene co-segregated.