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Genetic Engineering of Dunaliella: Potential for Improved Biofuel Production.

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Abstract:

Biodiesel produced from algal lipids is a promising source of renewable energy. Reasons for using algae, as opposed to vascular plants, are numerous. Oleaginous algae do not require fertile land or fresh water to grow, and therefore do not compete with food crop resources. Algae have short generation times and higher growth rates than larger vascular plants allowing for synthesis and accumulation of large quantities of neutral lipids, about 20-50% of the dry cell weight, in a short period of time. The efficiency of biodiesel production from oleaginous algae could be improved by using transgenic (genetically engineered) algae. The goal of this research is to develop techniques for transforming algae species from the genus *Dunaliella* to increase its carbon sequestration capability and lipid biosynthesis. It has been learned in our lab that increased biosynthesis of polyamines can positively influence carbon sequestration; other labs have shown that enzymes involved in triacylglycerol (TAG) synthesis can enhance lipid production. To genetically engineer *Dunaliella*, in addition to genes, promoters are also needed to regulate the expression of these genes. Currently we are working on cloning the promoters for highly expressed genes, i.e. actin, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), and light-harvesting complex II apoprotein (cab4). These promoters will be recombined with the polyamine biosynthetic genes already cloned and introduced into the algal cell for a high degree of expression.

Introduction and Background on Algae:

The word algae is used to describe a wide range of phototrophs that carry out oxygen-evolving photosynthesis, but differ from other phototrophic organisms in that they lack phloem (food conducting tissue), xylem (water conducting tissue), and multicellular sex organs surrounded by sterile cells (Rosenberg et al 2008). Both unicellular green algae and red algae are eukaryotes that evolved from a common endosymbiosis, where a heterotrophic cell engulfed and incorporated a photosynthetic cyanobacterium. Red algae and green algae differ in the photosynthetic pigments they use. Red algae contain chlorophyll *a* (chl *a*) but not chlorophyll *b* (chl *b*). Additionally, red algae contain phycobiliproteins that lend them their characteristic red color and allow them to utilize shorter wavelengths of light. Green algae are the ancestors to modern plants. Like modern plants, they have both chl *a* and chl *b* but lack phycobilins. There are two main groups of green algae, chlorophytes and charophyceans. Chlorophytes exhibit a large variety of morphologies; organisms in this group may be unicellular, filamentous, colonial, or multicellular. Most Chlorophytes have a complex life cycle with both sexual and asexual reproduction. These versatile organisms are found in nearly every imaginable habitat where light is present. Endolithic phototrophs can even grow inside small spaces in porous rocks in desserts and in the Antarctic. Chlorophytes play an important environmental role and are the primary producers in many ecosystems (Madigan et al 2009). Chlorophytes are also important economically and are used in a variety of ways. Chlorophytes are used as food for people and live stock as well as for fertilizing crops. Organic compounds produced by chlorophytes are used

in a number of industries including food processing, cosmetics, pharmaceuticals, nutraceuticals, textiles, paper, and printing (Rosenberg et al 2008).

Success of Algae in Industry and Potential for Biofuel:
Part of the reason these organisms are able to exist in such a wide range of habitats is that they have very short generation times when compared to more complex vascular plants. This allows them to adapt and diversify quickly. The small size and short generation time of many chlorophytes makes them efficient at converting solar energy into useful metabolites (ESCAP, APCAEM, 2008). Oleaginous algae can synthesize and accumulate a large quantity of neutral lipids, about 20-50% of the dry cell weight, in a short period of time. Based on the photosynthetic efficiency and growth potential of algae, theoretically about 200 barrels of algal oil could be produced per hectare of land (Hu et al 2008). Additionally, chlorophytes do not require fertile soil to be grown so they do not compete with food crops (ESCAP, APCAEM, 2008). Currently, soy, a valued food crop, is used to produce biodiesel in the US. If land used to produce biodiesel from soy were instead used to produce algal oil, the oil produced would be 100 times greater. Other benefits of algae include, carbon sequestration, ability to utilize nutrients in agricultural runoff and municipal wastewater, and the added revenue gained from the production of secondary metabolites (Hu et all 2008). For these reasons, many people have turned to chlorophytes as a means of producing biofuel.

Why Dunaliella:
Algae of the genus *Dunaliella* have many promising characteristics that make them well suited for biodiesel production. Cells of the genus *Dunaliella* lack cell walls made of cellulose. Less energy and carbon is used to build cellulose, and more is available for cell growth and lipid synthesis. Additionally, many laboratory techniques used to access or influence intercellular components are more difficult to perform on cells with thick cell walls (Wiley 2009).
Cells of this genus are easily grown and tolerate a wide range of conditions. Many are halotolerant or are halophiles. This allows *Dunaliella* to be grown without fresh water, a valuable resource (Oren 2005). This genus is also resistant to photobleaching. Many species of *Dunaliella*, including *D. salina* and *D. tertiolecta*, produce large amounts of inner-thylakoid beta-carotene. It is hypothesized that this pigment may help prevent damage to photosensitive parts of the photosystems. This could allow these species to be grown more efficiently on a large scale where controlling the exact amount of light may not be feasible. Physiological damage, such as photobleaching, can result from exposure to light intensities to high to be absorbed. The D1 protein in photosystem II can be damaged by intense light causing photoinhibition (reduced photosynthetic rates) and reduced growth and production (Wiley 2009).
Beta-carotene not only protects cells from intense light, but it is also a valuable nutraceutical. Beta-carotene is a provitamin that is converted to vitamin A needed to form rhodopsin in the outer segment of rod cells in the eye. Beta-carotene also has antioxidant properties (Lawson et al 2007). Because conditions that increase the lipid content of some species of *Dunaliella*, such as *D. salina*, also increase the beta-carotene content, selling this product as a nutraceutical could provide another source of revenue for a biofuel company (Mendoza et al 2007).
Species of *Dunaliella*, such as *D. tertiolecta*, also produce large amounts of glycerol. Glycerol is produced to increase cytoplasmic solute concentrations to raise the osmotic pressure of the cytoplasm and prevent fluid loss in saline solutions. Glycerol concentrations in the cell can be as high as 8M (Wiley 2009). Glycerol is already produced by some biodiesel industries and

attempts are currently underway to find more ways to use this byproduct in industrial chemical processes (Pagliaro et al 2007).

Bringing Algal Lipid Production to the Commercial Scale: Genetic Transformation

Despite the promising benefits of using algae as a source of renewable energy, the high costs of producing biofuel from algal oil have prevented widespread success at the industrial level. One method for offsetting the costs associated with growing the algae and extracting the oil is to increase the amount of oil produced by an individual cell.

Oleaginous algae contain different amounts of lipids depending on their growing conditions. Under normal growing conditions, oleaginous green algae have a total lipid content of about 25.5%. The vast majority of these lipids are fatty acids that have undergone an esterification reaction to produce glycerol-based membrane lipids. Membrane lipids constitute 5-20% of the dry cell weight (DCW) of oleaginous algae. However, under stressful environmental conditions the average lipid content increases to 45.7% DCW. Under stressful conditions, algae stop growing and dividing and conserve energy by directing lipid biosynthetic pathways towards the formation of neutral lipids, primarily triacylglycerol (TAG). TAGs have no structural role in the algae and are primarily a storage form of carbon and energy. TAGs can be seen in densely packed lipid bodies located in the cytoplasm, or in the inter-thylakoid space of the chloroplast where they are often associated with Beta-carotene and are called plastoglobuli. Understanding the mechanisms that induce TAG sequestration in algal cells, and genetically manipulating them, could greatly increase lipid production, and potentially allow biofuel to be produced on a larger commercial level (Hu et al 2008).

All TAGs are synthesized by a single set of enzymes in the chloroplast, and Acetyl-CoA carboxylase (ACCase) is a key enzyme responsible for regulating TAG synthesis (see figure 1). Although more is known about the metabolic pathways of vascular plants, it is thought that the synthesis of fatty acids starts with glycolysis derived pyruvate. Glycolysis and pyruvate kinase catalyze the irreversible synthesis of pyruvate. Pyruvate is converted to acetyl CoA, which is the major photosynthate used to produce fatty acids. ACCase is the enzyme that catalyzes the primary reaction, the conversion of acetyl CoA to malonyl CoA, in the biosynthetic pathway used to create fatty acids (Hu et al 2008).

Further evidence to suggest that ACCase plays an integral role in TAG synthesis and the cell's response to environmental stress is that, in *Cyclotella cryptica,* ACCase activity is directly related to silicon deficiency stress. ACCase activity increased as the length of time cells were grown with limited silicon increased. It is likely that this increase in activity was due to an increase in enzyme synthesis, because the addition of enzyme synthesis inhibitors inhibited enzyme activity (Hu et al 2008).

Increasing the number of copies of the gene for ACCase a cell carries could cause that cell to transcribe and translate more ACCase and increase ACCase activity. An increase in ACCase activity could potentially lead to the production and sequestration of TAGs without subjecting the cell to stressful stimuli (Dunahay, 1996). This is one method of genetic manipulation that could help to make growing algae for biofuel more profitable.

Figure 1: Fatty Acid de novo synthesis pathway in chloroplasts (Hu et al 2008)
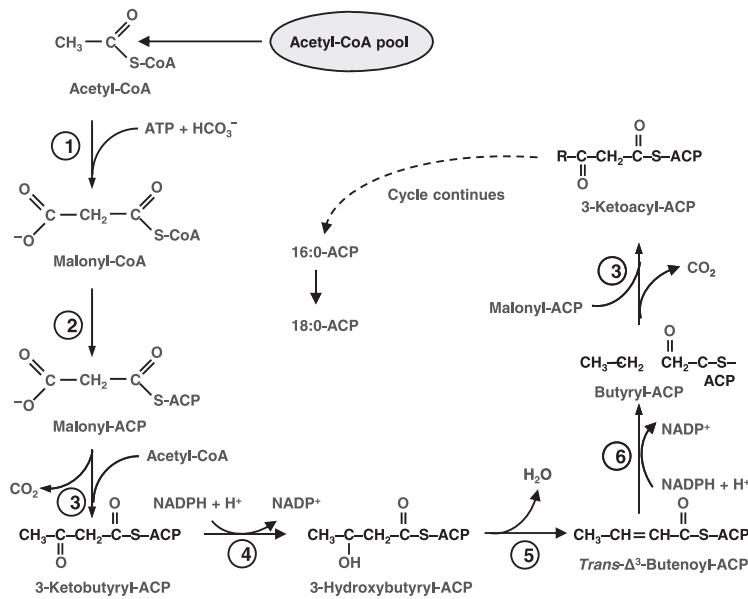
Acetyl CoA enters the pathway as a substrate for acetyl CoA carboxylase (Reaction 1) as well as a substrate for the initial condensation reaction (Reaction 3). Reaction 2, which is catalyzed by malonyl CoA:ACP transferase and transfers malonyl from CoA to form malonyl ACP. Malonyl ACP is the carbon donor for subsequent elongation reactions. After subsequent condensations, the 3-ketoacyl ACP product is reduced (Reaction 4), dehydrated (Reaction 5) and reduced again (Reaction 6), by 3-ketoacyl ACP reductase, 3-hydroxyacyl ACP dehydrase and enoyl ACP reductase, respectively (adapted and modified from Ohlrogge and Browse, 1995).

Although influencing the lipid synthesis pathway in the chloroplast is one possible technique for improving lipid production, there is a drawback to this approach. There is no guarantee that altering ACCase production will have a profound effect on lipid synthesis. The assumption of this approach relies on the classical definition of a rate-limiting step adapted from Blackman's first definition in 1905: All metabolic pathways are regulated by a rate limiting step, the step is often regulated by negative feedback and is influenced by metabolites other than the direct products, altering enzymes involved in the rate-limiting step has a profound effect on production, and other enzymes in the pathway are near equilibrium and altering their concentrations does not have a large impact on production.

However, beginning in the 1970s another model of how enzymatic pathways are regulated has gained acceptance; many enzymes control the rate of a reaction and altering one alone may only have a small impact (Thomas and Fell 1998). According to this view, improving lipid production will likely necessitate altering expression of many enzymes involved in the lipid synthesis pathway.

An approach to overcome this problem is to increase production of a secondary metabolite, such as a polyamine, that influences many metabolic processes, rather than targeting a single enzyme in a pathway. Polyamines have been found in all living organisms and influence a variety of processes in plants including cell division, reproductive organ development, root growth, flowering, fruit ripening, leaf senescence, and responses to environmental and pathogen induced stress (Kaur-Sawhney et al 2003, Galston and Sawhney 1990). Previous experiments in the Minocha lab at the University of New Hampshire, Durham, found that increasing polyamine biosynthesis genes (i.e. ornithine decarboxylase) increased carbon sequestration in higher plants (Mohapatra et al 2010).

This indicates that improvements in lipid synthesis in oleaginous algae may be achieved by increasing polyamine biosynthesis genes as well as gees involved in TAG synthesis. It is possible that increasing polyamine biosynthesis genes could increase the carbon in the cell available for catabolic processes and increasing genes involved in TAG synthesis could push catabolic processes towards lipid synthesis.

Project Goals

The goal of this research project was to develop a procedure for transforming species of Dunaliella in order to pave the way for future work on genetic engineering of Dunaliella to improve lipid synthesis.

Experimental Design

Genetic engineering requires four parts: 1) a transformation method, 2) a selection method, 3) a promoter that induces high expression, and 4) a reporter gene. Development of each of these four components is discussed below. For a summary table of transformation conditions and the selection method see tables 1 and 2.

Transformation Method

The four primary techniques for algal transformation include particle bombardment, electroporation, glass beads, and Agrobacterium tumifactiens-mediated method. Each method has its advantages and disadvantages, but all transformations follow the same general steps:

1. Introduction of DNA through one of the above methods and incorporation of exogenous DNA into the cell, either as part of the genome or as episomal DNA
2. Selection of successfully transformed cells by:
    a. Growing cells on a medium with herbicides or antibiotics (ex: Phosphinothricin, hygromycin) wildtype cells cannot survive in.
    b. Producing auxotrophic transformants and then transferring those transformants to a medium with the required nutrients.
3. Means of quantifying the strength of a promoter:
    a. Adding the gene that codes for Beta-glucuronidase and staining with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) causes transformed cells appear different shades of blue depending on the strength of the promoter; a darker blue color indicates a stronger promoter
    b. Adding the gene for green fluorescent protein, which causes transformed cells to fluoresce different intensities depending o the strength of the promoter; brighter fluorescence indicates a stronger promoter.

For this experiment, electroporation was chosen as the method for transformation because it has been successfully used with *Dunaliella* (Geng et al 2004, Sun et al 2005, and Feng et al 2008 to name a few), it is appropriate to use with this genus because *Dunaliella* cells lack tough cell walls, the equipment needed was readily available, it is inexpensive, fast, and easy. Electroporation incorporates exogenous DNA by exposing microalgae coated with exogenous DNA to an electrical field. This causes pores to open in the cell membranes and allows DNA to enter the cell. This method can result in low cell viabilities if the electric field is too strong or if an inappropriate liquid medium is used for electroporation.

According to previous experiments, maximum transformant yields were reported with an electric field intensity of 1,000 to 1,800 V/cm for pulse durations of 2-26 ms. Cuvette used varied in width from 0.2 to 0.4 cm with volumes between 100 to 800ul. A wide range of cell concentrations were used in previous experiments, ranging from 0.2 to 400 million cells/ml (Coll 2006). For conditions used in this experiment, refer to table 1.

One of the most important factors when using electroporation is the electroporation buffer. Because some of the medium enters the permeabilized cells, the medium must have a similar osmotic pressure to the cytosol and must not contain molecules that are harmful to the

cell. Liquid mediums generally contain 10-30 mM HEPES or Tris buffer to maintain the pH near physiologic levels, 5-10 mM $CaCl_2$ to neutralize the negative DNA charges so that DNA binds better to microalgae membranes, and 0.04-0.4M sucrose, sorbitol, and or mannitol to maintain osmolarity and increase the survival of the cells (Coll 2006). In this experiment, the electroporation buffer was made according to Sun et al 2005 (see Appendix for buffer contents). Cells were washed three times in the electroporation buffer to remove excess salt prior to electroporation. After each wash, a small sample of cells was plated on 1.5% agar and TK media to ensure that cells remained viable throughout the wash cycles prior to electroporation.

Immediately following electroporation cells were cooled on ice for 5min to minimize damage caused by shocking. A small sample of these cells was also plated and the remainder was transferred to chilled 1.5ml microcentrifuge tubes containing 1ml of TK media. The cells were grown on shakers under 12 hour light/dark cycles for two days before staining with X-Gluc for eight hours at 37^0C and de-staining with 70%ethanol.

Cells to be used in transformation need be harvested in log phase growth. Cells in logarithmic growth are more frequently undergoing mitosis so a larger portion of cells lack a nuclear envelope, which makes incorporation of exogenous DNA easier. Cells in logarithmic growth are also of a similar size and composition, and this increases the repeatability of the experiment. For a definition of log phase growth and the growing conditions used see the sections below.
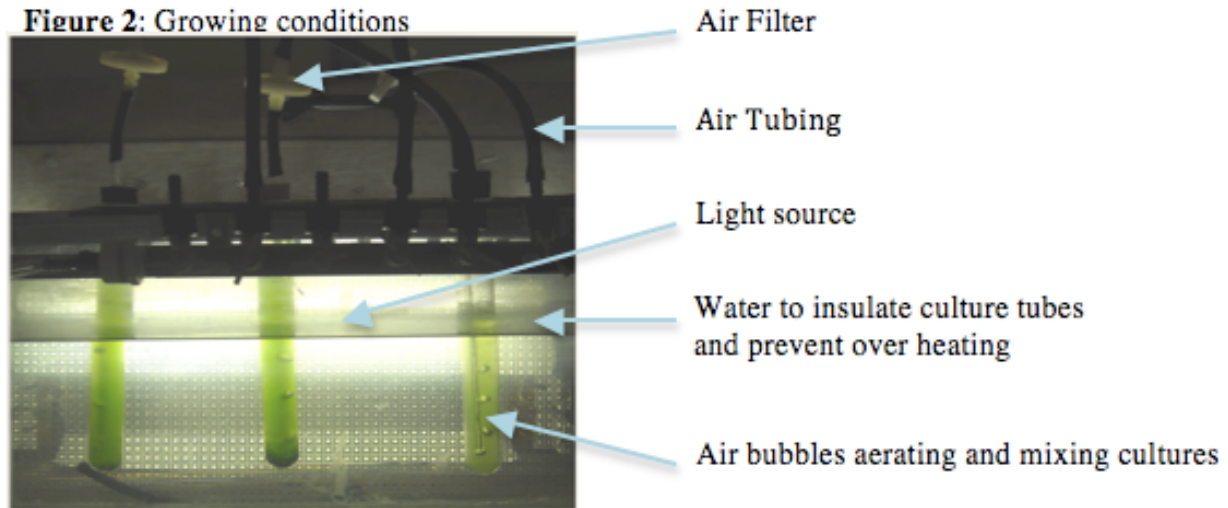
Table 1: Electroporation Conditions

Plasmid Name	Plasmid DNA (ng)	Number of cells/Cuvette	Volt Setting (V)	Actual volts (V)	V/cm Delivered	Cuvette width (cm)	Approximate Final Vol. (ul)
pCW122	500	$4.67*10^7$	250	180	900	0.2	50
pCW122	1,000	$4.67*10^7$	250	150	750	0.2	50
pCW122	500	$4.67*10^7$	400	230	1150	0.2	50
pCW122	1,000	$4.67*10^7$	400	200	1000	0.2	50
SPDS1-B1 in PMDCI63	500	$4.67*10^7$	250	140	700	0.2	50
SPDS1-B1 in PMDCI63	1,000	$4.67*10^7$	250	170	850	0.2	50
SPDS1-B1 in PMDCI63	500	$4.67*10^7$	400	230	1150	0.2	50
SPDS1-B1 in PMDCI63	1,000	$4.67*10^7$	400	200	1000	0.2	50

Growing Conditions

Three species of *Dunaliella*, *D. salina*, *D. bardawil*, and *D. tertiolecta*, were used in this experiment. Cells were grown in glass culture tubes with TK media (see appendix for media composition) at room temperature under continuous illumination with white light. Cells were continuously mixed and aerated by pumping filtered atmospheric air down a glass tube into the cultures (see figure 2). Under these conditions, log phase growth was generally achieved in five to seven days. Cells in log phase growth had between $4*10^6$ to $6*10^6$ cells/ml; this correlates to an optical density of 0.6-0.9 at λ687nm. Cultures were maintained by replacing old media weekly. Spent media was replaced by removing 5ml of the cells and old media, rinsing the

culture tubes with deionized water and then with TK media, adding 20ml of fresh TK and returning the 5ml of cell suspension. Dirty culture tubes were replaced and cells were washed bimonthly or as needed to remove small molecular weight contaminants (i.e. bacteria, metabolic waste products, etc). During cleaning, 10ml to 20ml of cell suspension was briefly centrifuged, and resuspended in fresh TK media three times before being transferred to a new culture tube with 20ml of TK.

Figure 2: Growing conditions

- Air Filter
- Air Tubing
- Light source
- Water to insulate culture tubes and prevent over heating
- Air bubbles aerating and mixing cultures

Selection Method

Three different antibiotics commonly used with higher plants, hygromycin, kanamycin, and chloramphenicol at concentrations ranging from 5ug/ml to 300ug/ml were tested with all three species. Antibiotic plates were made by heating 1.5% agar in TK media to dissolve the agar. Once the media cooled to about 55^0C, the antibiotic was added and thoroughly mixed. After the plates had completely cooled, an autoclaved piece of filter paper (grade fine) was placed on each plate (see figure 3). Using filter paper allows colonies to be easily transferred to a new plate, and it makes visualizing distinct colonies easier. Distinct colonies were achieved when 200ul of diluted cell suspension was used to inoculate each plate. *D. tertiolecta* was diluted to 0.6 OD_{687}, *D. salina* was diluted to 0.3 OD_{687}, and *D. bardawil* was diluted to 0.35 OD_{687} prior to plating.

Figure 3: Example antibiotic test results after 10 days of growth

| *D. Salina* Control | *D. Salina* + 300 µg/ml Hygromycin | *D. salina* + 100 µg/ml Chloramphenicol |
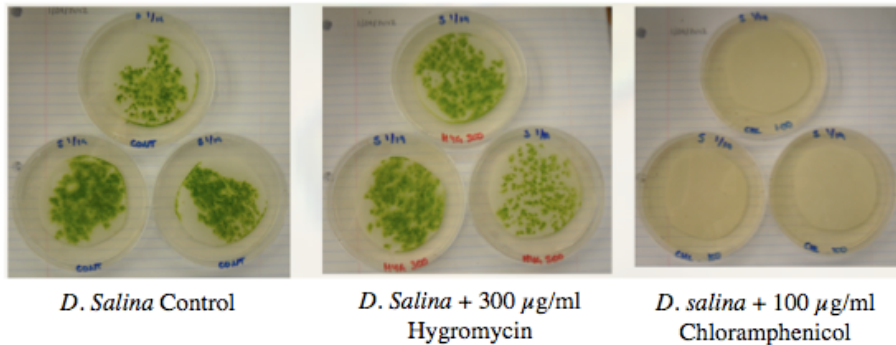
Highly Expressed Promoter

Both native and foreign promoters were tested in this experiment. The Two foreign promoters tested were ones successfully used in previous experiments with higher plants in the Minocha lab, spermidine synthase promoter (SPDS1) in plasmid vector backbone PMDC163 and the cauliflower mosaic virus 35S promoter (CaMV35S) in plasmid vector backbone pCW122. CaMV35S has been used successfully in other labs to induce expression of foreign genes in species of *Dunaliella* (Coll 2006).

In order to clone native promoter regions from all three species, genomic DNA was extracted using two different techniques. Initially the CTAB method adapted from the procedure used by Stewart et al (1993) was used. Later, this method was replaced by the TaKaRa Plant DNA Isolation Kit, which took less time and yielded higher concentrations of DNA according to evaluation with the NanoDrop. Genomic DNA was isolated according to the manufacture's instructions except that 0.05g of PVPP per gram of tissue was added in step one to remove any pigments.

Seven primers based on different promoter sequences for all three species found using GenBank were used to PCR amplify genomic DNA for all three species (for primer sequences and related notes, see appendix). RubyTaq Mastermix (2x) was the DA taq polymerase used. The PCR settings were designed according to the manufacture's instructions, and all samples were run on a gradient from 45 to 65^{0}C. Only the primers designed from genomic DNA from *D. tertiolecta* for the promoter region of the light-harvesting complex II apoprotein (cab4) gene were successfully PCR amplified. For this reason, only this promoter was used in the rest of the experiment.

The PCR product for the cab4 promoter was gel purified and ligated into a pCR8/GW/TOPO vector backbone. This plasmid was used to transform competent *E. coli* cells via heat shocking. *E. coli* cells were allowed to grow overnight on plates with spectinomycin (spec). Distinct colonies were selected from each plate and used to inoculate liquid LB media + spec. After an overnight incubation at 37^{0}C on shakers, plasmid DNA was isolated using the Promega Magic Miniprep Kit and the results were analyzed using a restriction digest with EcoRI (see figures 4 and 5 for PCR and EcoRI digestion results).

Reporter Gene

The reporter gene used in this experiment was beta-glucuronidase (Gus). Both the PMDC163 and pCW122 plasmids contained the Gus gene. The enzyme beta-glucuronidase is not natively expressed in plants, and cells expressing Gus turn blue in the presence of X-Gluc stain (Jefferson 1987). Gus has been used in a previous experiment with *D. salina* to demonstrate successful expression of exogenous DNA (Feng et al 2008). This reporter system was chosen because the blue color expressed allows for quantitative evaluation of promoter driven expression. Growing transformed cells on antibiotic only allows qualitative analysis of gene expression. If cells grow, exogenous DNA is being expressed; if not, transformation was not successful. The Gus reporter system produces a gradient of blue color; a darker blue color directly correlates with more gene expression. This can be used to determine the most appropriate promoter to induce gene expression.

Results

Under the conditions described above, observations of the plates showed that cells survived all three washes and electroporation. Also, cells growing in 1ml of TK media survived after two days. This was confirmed by observing the cells using a light microscope at 100X magnification.

Successful transformation was not confirmed because both staining the cells with X-Gluc and destaining with 70% ethanol lysed the cell. When stained cells were observed under a light microscope at 100X magnification, it was difficult to confirm if any localized blue color was present. There were areas of darker color, but it cannot be definitively stated that this is evidence of successful transformation.

To address this issue, methods for cell immobilization were tested. Preliminary tests showed that cells grown on a thin layer of 0.8% agar on a slide could be clearly seen under the microscope (see figure 5). Additionally, if the slide was kept under 100% humidity, cells grew well on the slide for at least five days with 12-hour light/dark cycles (see figure 4). Once the cells had been growing on the slide for eight hours, they became fixed and didn't move after destaining with 70% ethanol (see figure 5).
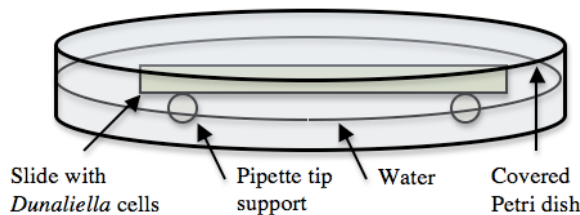

Slide with
Dunaliella cells   Pipette tip   Water   Covered
                        support   Petri dish

Figure 4: Growing conditions used to maintain 100% humidity. The agar coated slide with Dunaliella cells was kept above the surface of the water using pipette tips.
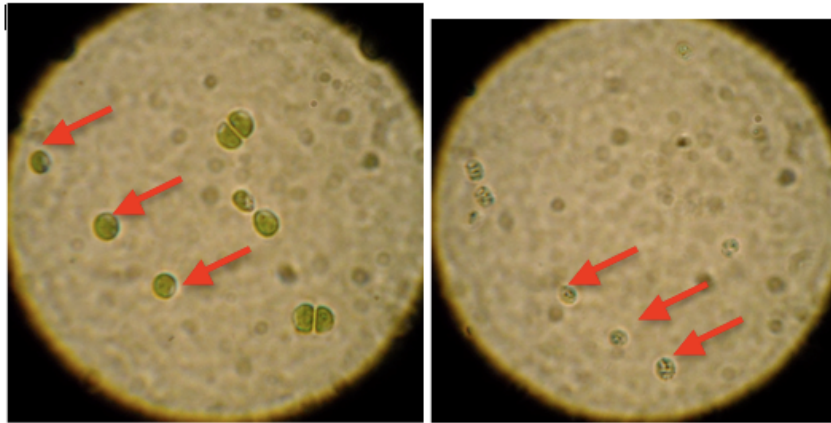

Figure 5: D. salina cells after growing on an agar coated slide for eight hours. The three arrows point to the same three cells of D. salina before and after destaining with 70% ethanol. The cells on the right appear smaller because they have lysed. Both pictures were taken with 100x magnification.

The antibiotic tests showed that species of *Dunaliella* are highly resistant to both kanamycin and hygromycin. Only chloramphenicol had an effect on cell growth. For a summary of antibiotic test results, see table 2.

Table 2: Summary of antibiotic test results

Species	Antibiotic	Results
D. salina	hygromycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	kanamycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	chloramphenicol	Antibiotic concentrations between 5-300ug/ml were tested. Even at the lowest concentrations used, no cells grew.
D. bardawil	hygromycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	kanamycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	chloramphenicol	Antibiotic concentrations between 5-300ug/ml were tested. Even at the lowest concentrations used, no cells grew.
D. tertiolecta	hygromycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	kanamycin	Concentrations between 25-300ug/ml were tested. Cell growth was not effected by the antibiotic even at very high concentrations.
	chloramphenicol	Antibiotic concentrations between 5-300ug/ml were tested. At the lowest concentration, 5ug/ml, cell growth was only slightly inhibited. At a concentration of 25ug/ml cell growth was much slower than the control. At a concentration of 100ug/ml no cells grew.

PCR amplification of genomic DNA was successful when primers designed for the promoter sequence of cab4 (*D. tertiolecta* genomic DNA) were used. Through running the reaction on a gradient it was determined that the best results were achieved with an annealing temperature of 54^0C. Based on the reported sequence for this promoter (GenBnak: U28725.1) the expected product length was 486pb. A band of this length was consistently achieved for D. tertiolecta. A band of roughly 300bp was seen in both D. bardawil and D. salina (see figure 6)
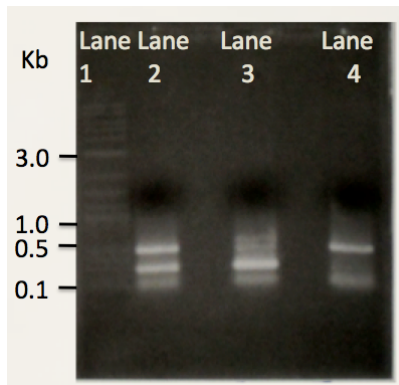

Figure 6: This is a gel of the PCR products from *D. bardawil* (lane 2), *D. salina* (lane 3), and *D. tertiolecta* (lane 4) using the primer for the for the cab4 promoter region. A band of the expected length (486bp) is seen in lane 4, and an additional unidentified band (of about 300bp) is seen in lanes 2 and 3

The PCR products in figure 6 were gel purified, ligated into a TOPO vector, and used to transform *E. coli* cells. After transformation, plasmid DNA was isolated from 10 colonies from three separate plates corresponding to lanes 2, 3, and 4 in figure 6. A restriction digest using EcoRI (cut sites at 671 and 1175bp) followed by gel analysis was used to determine if colonies had the correct insert. The results indicate that cloning was successful. Several colonies had the insert of the expected length, and some had the unknown insert (See figure 7 and table 3).
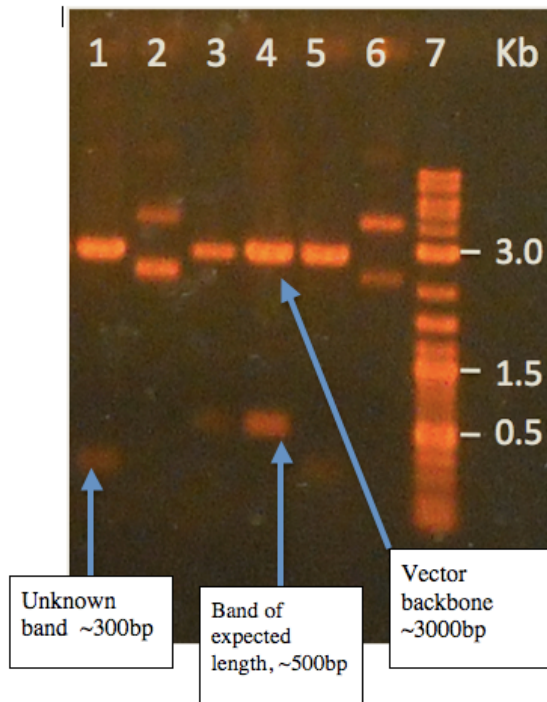
Figure 7: This is a section of the gel used to analyze the EcoRI restriction digest. This section of the gel corresponds to plasmid DNA isolated from E. coli transformed with a TOPO vector containing inserts from lane 2 in figure 6.

Table 3: Summary of results of EcoRI restriction digest

Possible Sequence ligated into pCR8/GW/TOPO	Expected product size for successful ligation (bp)	Plate Corresponding to lane 4 in figure 6	Plate corresponding to lane 3 in figure 6	Plate corresponding to lane 2 in figure 6
Cab4 promoter	2799 and 504	6/10 had cab4 promoter insert	0/10 had the cab4 promoter insert	6/10 had the cab4 promoter insert
Unknown sequence of about 300bp from lanes 2 and 3 in figure 6	2799 and approximately 300	0/10 had the unknown insert	2/10 had the unknown insert	2/10 had the unknown insert

Discussion

Although conformation of successful transformation was not achieved, based on the cell survival rate and previously published studies, it is likely that this transformation protocol could be used to transform *Dunaliella* species (Geng et al 2004, Sun et al 2005, and Feng et al 2008). Preliminary tests using a higher voltage setting (1,000V) and different post electroporation growth conditions (cells were added to 50ml TK instead of 1ml) resulted in 100% cell death (data not shown). Using the adjusted protocol, cells survived all steps including the three pre electroporation washes. It should be noted that when fewer washes were done, too much salt was carried over from the TK media into the electroporation buffer; this caused arching and prevented accurate delivery of the desired voltage. Carefully performing all three washes prevented this problem.

Conformation of transformation was not achieved because the Gus reporter system needs to be adapted for use with *Dunaliella* species. *Dunaliella* species lack tough cell walls. This makes transformation and accessing intracellular components easier, but it led to lysis during staining. Preliminary tests using agar to immobilize the cells suggests that this may be used to fix

cells during staining to allow visualization of localized blue color even if cells lyse. Previous experiments using agar to immobilize yeast found that cells could be mixed with cooled agar (45-50^{0}C) and then pressed into a thin layer (Gad and lkai 1995, and Lebeau et al 1998). These cells were possible to visualize using confocal microscopy. In both experiments cells survived immobilization and Lebeau et al (1998) found that nutrients and waste products could diffuse through the agar matrix. It is possible that a similar technique may be used to immobilize Dunaliella cells prior to staining with X-Gluc. However, tests are needed to determine if the X-Gal substrate can diffuse through an agar matrix, before this technique is used.

Confirmation of successful transformation may also be achieved by growing the cells in a selective environment post electroporation. Antibiotic tests revealed that chloramphenicol resistance is an appropriate selectable marker. The gene encoding chloramphenicol acetyltransferase (CAT) confers resistance to this antibiotic. Promoters of interest could be evaluated by using them to drive expression of CAT. However, this system is not as sensitive as the Gus reporter system.

The results of the EcoR1 digest shown in figure 7 indicate that cloning of the cab4 promoter was successful. An experiment by Escoubas et al in 1995 revealed that expression of light-harvesting chlorophyll protein complex apoproteins associated with photosystem II was controlled at the level of transcription in response to different light intensities. When *D. tertiolecta* cells were moved from high light intensity (700 ,mol of quantam-2s-1) to low light intensity (LL; 70 ,umol of quanta-m-2.s-1) after nine hours, there was a 3-4-fold increase in cab mRNA. This suggests that expression of genes regulated by the cab4 promoter could be controlled by altering the light intensity. Having the ability to influence gene expression by altering the growing conditions may be useful for improving lipid production by *Dunaliella* species.

Although further research into this topic is needed, the results of this experiment suggest that the transformation protocol used results in a high survival rate, the use of agar to immobilize the cells may lead to the ability to visualize the cells after staining, the CAT gene is an appropriate selectable marker, and the cab4 promoter was successfully cloned. Future research should focus on improving the X-Gluc staining procedure and designing a plasmid using cab4 to regulate expression of CAT and Gus.

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