Transformation, Growth, and the Cytoskeleton:

Tools to Study Oil Producing Algae

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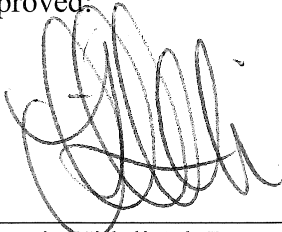

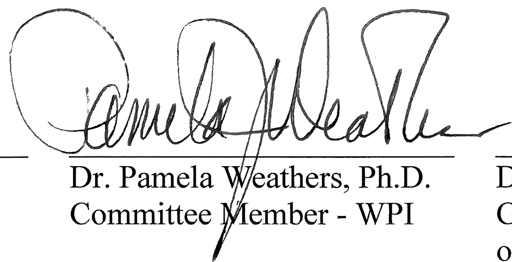

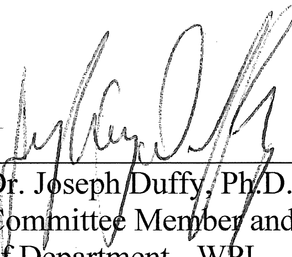

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Abstract

With the current state of climate change and world peak oil on the horizon, it is important to focus our research efforts on alternative sources of energy. Ethanol obtained from the digestion of biomass (bioethanol) and oil harvesting from algae (biodiesel) are two promising fields of study for transportation fuel production. However, in their current state of development, neither option is capable of reasonably replacing the transportation fuel demand for this country. The land demand needed is too large for either process to become a viable option, albeit the land demand for biodiesel is considerably smaller than that of bioethanol. Therefore, when moving forward with alternative transportation fuel, harvesting oil from algae is a more promising option. Therefore, I investigated oil producing green algae to better understand algal growth, the algal cytoskeleton, and tried to establish a methodology to genetically manipulate algae.

I developed a microgrowth assay in order to investigate algal growth and proliferation, while at the same time using considerably less material and space. This assay can directly monitor algal growth in response to media contents, and overcomes many of the limitations of existing microassays due to its use of solid media agar and fluorescent imaging. I also investigated algal genetic manipulation with the intention of creating a standard operating procedure, which could lead to further investigation of how to increase lipid output and increase lipid harvesting cycles through studying lipid production and cell division. Electroporation and PEG mediated transformation were the two chief methods investigated for nuclear transformation. Lastly, I performed an algal kinesin phylogenetic study to characterize the currently available algal kinesin superfamily, providing insight to proteins that are important for cell division as well as other functions within this superfamily. Kinesins 5, Kinesin 7s Class II and Class V, and Kinesin 14 Class I were identified to be important for algal cell division, while Kinesin 8, 12, 11, and some orphan kinesins will require further investigation due to their unknown plant function. Overall, this research provides a foundation for future algal studies required for optimal oil production necessary for a more sustainable future.

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Chapter 1: Introduction

As of 2010, 19.1 million barrels of oil are used daily in the United States with over 14 million barrels dedicated to transportation fuel (British Petroleum, 2011). This rate of consumption is currently leading to a rapid depletion of fossil fuels and an excessive release of carbon into the atmosphere. Therefore, alternative energy sources are necessary to supply the current state of energy consumption as well as maintain a healthy environment. Most assessments of the world's oil reserves suggest that peak oil, the point at which maximum oil production is reached and a terminal decline of production will begin, has been or will be attained within this decade (Koppelaar, 2005, Cohen, 2007, Zittel & Schindler, 2007, Owen *et al.*, 2010). There are more optimistic predictions suggesting that the world's peak oil may not be reached until after 2020 (Koppelaar, 2005). However the facts remain the same, peak oil will be reached relatively soon and oil is not an unlimited fuel source. Furthermore, removing carbon from the ground and releasing it into the atmosphere has led to a current atmospheric CO_2 concentration of 397 ppm, which is a 113 ppm increase in CO_2 levels since the preindustrial times of the 1800s (Tans, 2012). Moreover, with CO_2 suggested to be a major player in global warming and climate change it is important to move away from fossil fuels as energy sources and start investigating alternative forms of energy (Climate Research Board, 1979, Florides & Christodoulides, 2009). Thus, development of clean alternative fuel sources capable of ending our dependence on fossil fuel for transportation could be an important step towards a sustainable and environmentally friendly future.

The sun is the most reliable energy source for this planet. Therefore, from a biological perspective, photosynthetic biomass is the most logical source of alternative energy that should be investigated for fuel. The two most promising processes of producing liquid transportation

fuel from biomass are fermentation of sugars to produce ethanol and harvesting lipids from microalgae to create biodiesel. While both of these processes offer some promise, they need to be better understood and optimized in order for them to become commercially viable (Jones & Mayfieldt, 2012).

Bioethanol is produced from either yeast or bacteria cultures that break down sugars to produce ethanol. The most common organisms investigated include *Escherichia coli*, *Clostridium* sp., and *Saccharomyces* sp. while the most studied sources of sugars are sugarcane, maize, switchgrass, wood chips, and stovers (Sanchez & Cardona, 2008). The generic process of producing bioethanol involves mechanically milling the biomass, processing the sugars with chemicals or heat to saccharify the biomass, fermenting with the desired organism or culture, and finally distillation (Jordan *et al.*, 2012). The major setbacks of this process are that grain sugars by themselves do not produce enough ethanol to fuel the country and the current methods used to delignify cellulose are not economically affordable (Sanchez & Cardona, 2008, Jordan et al., 2012, Akinci *et al.*, 2008, Zamboni *et al.*, 2009). Additionally, the chemicals used to break down the lignin can cause problems with fermentation because residual compounds frequently left behind are toxic to the organisms used for fermentation (Bak *et al.*, 2009). Moreover, completely removing the toxic chemicals or using heat for further breakdown will result in inefficient techniques that are, again, not economically viable at a large commercial scale (Bak et al., 2009). These pitfalls do not render bioethanol production impractical because in the United States, the Air Force and many companies are invested in studying ethanol production from cellulosic biomass.

The alternative to bioethanol production is biodiesel production from oil producing plants and algae. The most studied organisms for lipid harvesting are green algae with some examples

being *Chlamydomonas reinhardtii*, *Chlorella* sp., *Ettlia oleoabundans*, *Nannochloropsis* sp., and *Botryococcus braunii* (Scott *et al.*, 2010, Li *et al.*, 2008). With the exception of *B. braunii*, the general process of producing biodiesel works by growing a dense culture of algae, stressing the culture, harvesting the lipids, and then transesterfying the lipids (triacylglycerides) to yield hydrocarbons for biodiesel fuel (Figure 1.1) (Scott et al., 2010, Chisti, 2007). One of the most common way of stressing the culture for over production of lipids is by nitrogen deprivation; though, it has also been shown that *C. reinhardtii* will produce lipids in response to high temperature, salinity stress, and other algae can over produce lipids through pH, temperature, heavy metals, UV irradiance, and light irradiance stress (Sharma *et al.*, 2012, Yang *et al.*, 2012, Siaut *et al.*, 2011). *B. braunii* is an exception to this process because it will constitutively produce lipids and hydrocarbons without the need of an external stress (Metzger & Largeau, 2005). However, the downside to *B. braunii* is that the hydrocarbon chain compositions produced from these lipids are not ideal for efficient biodiesel fuel and *B. braunii* has a slow doubling time of 72 hours in comparison to other algae such as *Chlamydomonas* sp., *Chlorella vulgaris, or Nannochloropsis sp.*, which have doubling times of 10 hours, 20 hours, and 14 hours, respectively (Sheehan *et al.*, 1998, Dent *et al.*, 2001, Javanmardian & Palsson, 1992, Kilian *et al.*, 2011).

As for the pitfalls of producing biodiesel from algae, the majority of lipids produced by the cells are intracellular rather than extracellular; however, established commercial companies have filed patents for harvesting extracellular lipids demonstrating the capability to overcome this problem (Roessler *et al.*, 2009). Also, due to the glycerol byproduct from the transesterification of the triacylglycerides (Figure 1.1), the amount of glycerol produced if biodiesel were used to replace all transportation fuel could potentially become a problem if a

major market for glycerol is not established (Demirbas & Demirbas, 2011, Akinci et al., 2008). It has been estimated that if only 10% of the diesel demand replaced with biodiesel, then the US market for glycerol can be met (Akinci et al., 2008). Therefore, if 100% of diesel is replaced with biodiesel, then there is a high potential of significant glycerol waste. However, what is important is that these two pitfalls do not impede the production process unlike the bioethanol pitfalls, nor do these pitfalls make biodiesel production more expensive. Moreover, research has shown that if glycerol was fermented with anaerobic bacteria, ethanol yields could potentially further the viability of biofuels, allowing ethanol sales to mitigate the biodiesel expenses, and a glycerol market to be established (Yazdani & Gonzalez, 2007).

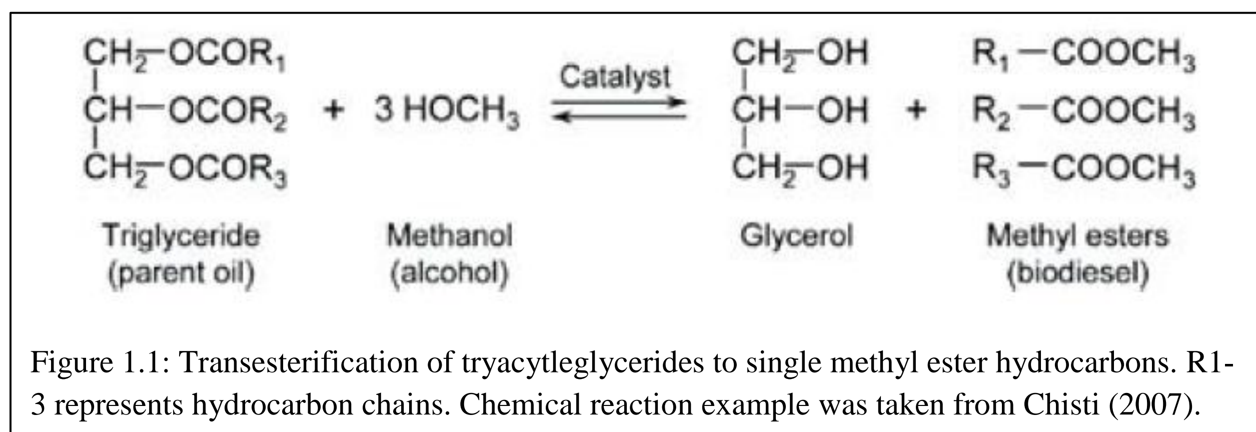

Figure 1.1: Transesterification of tryacytleglycerides to single methyl ester hydrocarbons. R1-3 represents hydrocarbon chains. Chemical reaction example was taken from Chisti (2007).

Although the biodiesel pitfalls are more favorable than the bioethanol pitfalls, neither method is useful unless the land demand needed to fuel the country is reduced. However, biodiesel is again more promising than bioethanol. For example, the United States is the world's largest producer of maize with 3.16×10^8 tonnes of maize harvested in 2010, which is over 1.7 times more maize than the second leading producer, China (Food and Agriculture Organization of the United Nations, 2010). According to a study performed by Akinci et al., in 2006, if the entire maize harvest was used to produce grain ethanol fuel, it would only replace 16.9-18.2% of the United States' gasoline consumption depending on the yield estimations (Akinci et al., 2008).

More problematic, however is that 501% of US land would be needed to replace all transportation fuel if fuel was produced from maize alone (Akinci et al., 2008). Conversely, in terms of biodiesel production from microalgae, and making some production assumptions, as little as 5% of the United States' land would be needed to produce enough fuel to replace all transportation fuel used in 2006 (Akinci et al., 2008). A similar conclusion was also made by Dismukes et al. in 2008 which grain ethanol or cellulosic ethanol cannot match the efficiency and smaller land demand of microalgae to fuel the country (Dismukes *et al.*, 2008). While this is more promising, the land demand is still large; 456,032 km^2 of US land would be completely devoted to harvesting lipids from microalgae to produce fuel (Akinci et al., 2008, Dismukes et al., 2008). Therefore, while both processes currently have a large requirements, biodiesel from algae is much more promising as it requires up to 100 fold less land to fuel the country.

In addition to the smaller land demand, biodiesel production is more promising because there are salt water algae that can produce lipids. For bioethanol digestion, both the organisms used and the most promising sources of digestible biomass are produced from freshwater sources. In contrast, biodiesel oil can be harvested from algae, such as *Nannochloropsis* sp., diatoms, and dinoflagates, which are all natural marine species and do not need the freshwater for fuel production. Therefore, if fuel was produced from the abundance of salt water available, the 2% of the world's water that exists as freshwater could be used for food production rather than divided between food and fuel.

In summary, biodiesel production from lipid producing salt water algae is most likely the best option for an alternative fuel source. Harvesting lipids can be optimized with no introduction of harsh chemicals or extreme physical conditions. Algae have a smaller land demand in comparison to bioethanol production. Furthermore, marine organisms circumvent the

debate of freshwater for food or freshwater for fuel. However, without higher density cultures and higher lipid output, biodiesel production will not become a viable option to maintain today's rate of fuel consumption because 5% of US land demand is still too high.

To address the problem of commercial viability of algae biodiesel production, it is crucial to increase the growth rate and the lipid output of oil producing algae. This would result in reduced land demand, while at same time trying to meet national fuel consumption rates. *Therefore, it is important that we develop a complete understanding of the basic mechanisms that control growth, proliferation and oil production in algae.* This requires that we develop cellular and molecular tools to standardize the study of these processes. These should include sensitive and reproducible assays for growth and proliferation, genetic manipulation to introduce novel genes or disrupt gene function, and a detailed molecular description of the cellular machinery responsible for cellular growth, proliferation, and oil production. To address some of these needs, in the present project I have 1) tried to establish a transformation protocol for some of the most commonly used oil producing algae, 2) developed a highly reproducible micro-growth assay for algae, and 3) I carried out a comparative phylogenetic analysis of algal kinesins, which are molecular motors that interact with microtubules. I believe that these projects will help provide information and tools that can be used to aid future algal research.

Chapter 2: Literature Review

2.1 Why Green Algae?

Green algae are the most viable option for mass producing biodiesel due to their high lipid output, relatively small land demand, marine species, and simple nutrient requirements. Microalgae such as *Chlorella* sp., *Ettlia oleoabundans*, and *Nannochloropsis* sp. have been shown to produce lipids up to 64%, 54%, and 68% of dry weight measurement when nitrogen starved, respectively (Chisti, 2007, Griffiths & Harrison, 2009, Demirbas & Demirbas, 2011). Algae produce these lipids in order to have energy reserves for when sunlight and nutrients are not available for the cell, the same way starch is produced and utilized by other photosynthetic organisms (Sharma et al., 2012, Radakovits *et al.*, 2010). These lipids are readily stored by the algae, and can be easily metabolized to provide energy for the cell, and are therefore overproduced in times of stress (Sharma et al., 2012, Radakovits et al., 2010). Over production of these lipids can be induced through a variety of culture stresses. Nitrogen deficiency and other kinds of nutrient limitations are the most commonly used method, while temperature, pH, salinity, and light irradiation stressors have also been investigated and shown to stimulate a lipid output increase (Sharma et al., 2012, Siaut et al., 2011, Radakovits et al., 2010, Yang et al., 2012). Furthermore, temperature stress could provide an efficient alternative route for lipid production as *Ettlia oleoabundans* has been shown to have a lipid output increase of 2.10 mg L^{-1} to 10.37 mg L^{-1} due to temperature increase from 25°C to 35°C with 130 mol photons m^{-2} s^{-1} of light (Yang et al., 2012).

Boytrococcus braunii has been shown to produce lipids up to 75% dry weight without the need of nitrogen removal, as it has the ability to constitutively deposit hydrocarbon chains within its cell wall (Metzger & Largeau, 2005). However, in relation to doubling time, *Chlorella*, *Ettlia*,

and *Nannochloropsis* are more useful organisms as they can divide under 24 hours, while *B. braunii* has a long doubling time of 72 hours, decreasing the efficiency of harvesting its lipids (Javanmardian & Palsson, 1992, Kilian et al., 2011, Metzger & Largeau, 2005, Chisti, 2008). Although, *B. braunii* is still an organism of interest and could be an important species for gasoline production as 67% of its hydrocarbon lipids can be carbon cracked for gasoline fuel (Hillen *et al.*, 1982).

These microalgal lipid outputs are much more promising for biodiesel production than other methods such as conventional oil producing crop plants, animal fat, or cooking oil waste, as there is just no practical means to harvest enough lipids. It is currently estimated that animal fats and oil crops can only displace 0.3% of the total current transportation fuel demand (Schenk *et al.*, 2008). For example, in terms of land demand, a conventional crop plant such as rapeseed requires 73% of the country's land to fuel the US transportation need, or on a larger scale, 55% of the world's arable land to fuel the planet, while estimates for microalgae need as little as 5% - 9% of the country's land to replace the US transportation fuel (Akinci et al., 2008, Schenk et al., 2008, Dismukes et al., 2008). Additionally, these crop sources still repeat the issue of freshwater for fuel or food as discussed in the introduction, as all of the crop plants suggested for lipid harvesting and even some of the microalgae are freshwater species. This becomes a problem if one of these species were used as the primary fuel source because the freshwater supply will be split between food and fuel, as discussed with bioethanol. However, there are marine organisms such as *Nannochloropsis* and diatoms which can avoid the debate and issues that arise with using freshwater for fuel or food.

In comparison to diatoms, green algae do not require an addition of silicon to the media in order to create its cell wall. This is important as the silicon is required for the diatom to

survive, it is another input for a fuel source, and it is more expensive than other nutrients required in F/2 marine media (Kroger & Poulsen, 2008). Furthermore, the necessity to add silicon into the media would be an economic burden that would hurt the viability of algal biodiesel fuel. Diatoms also tend to produce fewer lipids than green algae, as the average range of oil production is 22.7% - 37.8% of their dry weight (Hu *et al.*, 2008).

To realize the potential of algal biodiesel, green algae such as *Nannochloropsis* sp. should be used for further research. *B. braunii* has too slow of a doubling time to be used for efficient hydrocarbon harvesting, crop plants produce a much lower percentage of lipids per mass resulting in a very large land demand to fuel the country, and diatoms produce less oil and require more nutrients than green algae. Therefore, green algae should be focus on future biodiesel research with a concentration on marine green algae as they provide the best opportunity to create alternative liquid fuel from biomass.

2.2 Algal Transformation

Genetic manipulation, such as transformation, is a powerful tool that can be used in algae to modify and increase product output. The product output itself can range from lipids, antimicrobials, antioxidants, and other advantageous complex molecules (Guedes *et al.*, 2011). Furthermore, the autotrophic nature of microalgae provides a large potential to become an important bioreactor organism for the production of compounds and proteins over well-established systems such as *Escherichia coli* or *Pichia pastoris*. There have been 39 transformation protocols published on 26 different algae species, with only 13 of those species being green algae (Tables 3.1 and Appendix Table A). The methods published to transform the algae are agitation, particle bombardment, PEG mediated transformation, and electroporation.

Agitation, which consists of treating cells with autolysin and agitating them with glass beads or silicon carbide whiskers in the presence of PEG and DNA, has only been shown to work with *Chlamydomonas* due to its unique ability to regenerate and proliferate after the removal of its cell wall (Kindle, 1990, Dunahay, 1993). The transformation efficiency of agitation is 10 transformants per 10^6 cells per µg of DNA; however, due to *Chlamydomonas*' relatively low lipid content at 21% - 33% dry weight, it is not considered an ideal organism for biodiesel (Kindle, 1990, Dunahay, 1993, Griffiths & Harrison, 2009). Additionally, agitation is rarely used today for *Chlamydomonas* due to a more streamlined electroporation protocol with equivalent or better transformation efficiency of 17-200 transformants per 10^6 cells per µg of DNA (Brown *et al.*, 1991, Shimogawara *et al.*, 1998).

Particle bombardment, where DNA coated microparticles are bombarded into the cell's nucleus, produces only low transformation efficiencies microaglae. The transformation efficiency ranged from 0.75 to 16 transformants per 10^6 cells per µg DNA depending on the species (Dawson *et al.*, 1997, El-Sheekh, 1999, Jakobiak *et al.*, 2004, Kindle *et al.*, 1989, Schiedlmeier *et al.*, 1994, Tan *et al.*, 2005, Teng *et al.*, 2002). Additionally, as discussed in Chapter 3, particle bombardment can be a costly procedure and requires specialized equipment (Randolph-Anderson *et al.*, 1995). Moreover, in some cases some of the particle bombardment based transformations have displayed chloroplast transformation rather than nuclear transformation, which is due to the large size of the chloroplast in microalgae (Doetsch *et al.*, 2001, Lapidot *et al.*, 2002).

Although there are only two protocols published reporting PEG mediated transformation of microalgae, there are multiple protocols published describing protoplasting, which is a required first step for PEG mediated transformation (Jarvis & Brown, 1991, Maruyama *et al.*,

1994, Hawkins & Nakamura, 1999, Chen *et al.*, 2008, Yamada & Sakaguchi, 1982, Yamada & Sakaguchi, 1981). However, as my research shows in Chapter 3, and also reported previously, algae have an inability to regenerate and proliferate after the enzymatic removal of the cell wall (Sheehan et al., 1998, Aach *et al.*, 1978). These conflicting reports and my own experience with the inability to regenerate algal protoplasts suggest that this topic needs to be investigated in more detail.

The last and most promising transformation protocol for microalgae is electroporation. There are 11 published protocols in the literature (Appendix Table A), two of which are the now well established protocols for *Chlamydomonas* transformation (Brown et al., 1991, Shimogawara et al., 1998, Griffiths & Harrison, 2009, Sun *et al.*, 2005, Sun *et al.*, 2006, Huang *et al.*, 1996, Chen et al., 2008, Maruyama et al., 1994). Of the methods reported for *Chlorella* transformation, two involved a discontinued electroporator, which used unusual parameters consisting of a high salinity buffer and 2^{10} pulses per sample, while the other *Chlorella* method has never been cited from its publication and was not reproducible in my hands as described in Chapter 3 (Chen *et al.*, 2001, Wang *et al.*, 2007, Chow & Tung, 1999). However, a recently reported method for the transformation of *Nannochloropsis* is likely to become a reproducible protocol, as it uses reasonable and more typical parameters such as a single high voltage, high capacitance shock and a low ionic strength buffer. The reported transformation efficiency of this protocol is 2.6 transformants per 10^6 cells per µg of DNA (Kilian et al., 2011). Nevertheless, there are no citations of this method, but a second laboratory has obtained transformation using a similar protocol (Christoph Benning Lab, Michigan State University, Blair Bullard). Therefore, future efforts should be placed on optimizing this method to make it reproducible as it was not viable in my hands (see Chapter 3).

2.3 Toxicity and Growth Assays

Toxicity assays are important for determining environmental or chemical effects on an organism (Bhattacharya *et al.*, 2011). Most methods achieve assessment of toxicity through dose response testing a response to increasing levels of the toxin using expensive experimental animals such as mice, rats, and fish (Boorman *et al.*, 1999). However, advancements have been developed in order to design informative, small scale, and inexpensive toxicity assays. As a result, many methods have started using brine shrimp, bacteria, and plants to obtain useful results based on dose responses to toxins (Wang, 1991, Wanyoike *et al.*, 2004, Farré & Barceló, 2003).

In terms of studying eukaryotic photosynthetic organisms, whole plant toxicity assays can be useful in determining a dose response, but they necessitate ample materials and space to grow (Wang, 1991). Therefore, algal growth and toxicity assays have been used as a less expensive alternative, which also require less maintenance (Kviderova, 2010, Mecozzi & Oteri, 2011, Skjelbred *et al.*, 2012). In these assays, optical density measurements based in chlorophyll fluorescence provide quantitative information indicative of the health and the density as the culture grows over time (Kviderova, 2010, Mecozzi & Oteri, 2011, Lewis, 1995). However, without miniaturization of these assays, cultures can also occupy large spaces and require large volumes of materials. For instance, Mecozzi and Oteri in 2011 provide a toxicity assay that is performed in volumes as large as 250 mL per sample.

An initial attempt at miniaturizing liquid assays to 96 well plate volumes was reported by Kviderova (2010). However, as described in Chapter 4 this assay has the problems of evaporation and cell settling. These two problems lead to the requirement of sealing the plate, flooding it with CO_2 to maintain a carbon source after sealing and inaccurate optical density

readings due to nonhomogeneous cultures from a lack of agitation. In Chapter 4 I describe a new method that is able to avoid these problems by using solid media agar.

A simple, inexpensive, miniaturized microgrowth assay can be useful in combination with general molecular and cellular biology as the assay can be used to identify modes of action for toxic chemicals and help understand cellular processes with mutant, knockdown, or knockout organisms (Bhattacharya et al., 2011). A previous report using a method similar to Kviderova (2010) showed the ability to test temperatures, salinities, and light conditions resulting in a rapid experiment analyzing 390 growth conditions for the motile algae *Pseudochattonella farcimen* (Skjelbred et al., 2012). Moreover, a solid media based microassay for microalgae has the ability to test these same conditions without the need to be sealed and with a large variety of non-motile algae. Additionally it can economically screen chemical libraries, aid algal molecular biology research, and potentially be automated.

2.4 Plant Kinesin Phylogenetics

The kinesin superfamily consists of motor proteins that travel along microtubules. They are important in cell division, organelle transportation, flagella and cilia motility, and cytoskeleton organization (Reddy & Day, 2001, Richardson *et al.*, 2006, Miki *et al.*, 2005, Shen *et al.*, 2012, Lawrence *et al.*, 2004). Due to the high homology of their motor domains, most kinesins can be ordered into 14 families, Kinesins 1 -14. The current nomenclature for the different families was introduced in 2004; this was necessary due to the existing naming confusion in the field (Lawrence et al., 2004). The homology of each family extends across kingdoms and each class within a family tends to also have similar function (Miki et al., 2005).

Only a few phylogenetic studies have been performed in the past that analyze plant kinesins. Kinesins have been ordered for *Arabidopsis* (61 kinesins), poplar (52 kinesins), two

species of rice (45 and 41 kinesins), the red algae *Cyanidioschyzon merolar* (5 kinesins), the green algae *Chlamydomonas reinhardtii* (23 kinesins), and *Physcomitrella patens* (71 kinesins) (Shen et al., 2012, Richardson et al., 2006, Reddy & Day, 2001). These phylogenetic studies are important because they provide insight into the landscape of kinesins in plants and help determine their importance, hypothesize their functions, and design experiments to test these hypotheses.

Because understanding cellular growth and proliferation represents an important challenge for biodiesel algal research (Chisti, 2007), the kinesin proteins involved in these processes should be investigated to determine their function and regulation. Phylogenetic analyses provide the first step towards detailed molecular characterization. Furthermore, with the current advancements made in genome sequencing, more alga species are having their genomes fully sequenced, and the annotation of existing genomes, such as *Coccomyxa subellipsoidea* C169, *Chlorella variabilis* NC64A, *Nannochloropsis* sp., and *Chlamydomonas reinhardtii* are more readily updated (JGI, 2009, Blanc *et al.*, 2010, Jinkerson *et al.*, 2012, Pan *et al.*, 2011, Merchant *et al.*, 2007). To add to this phylogenetic and annotation research, in Chapter 5 of this work, kinesin proteins from several micro-algae were identified and added to the pool of kinesins used in the Shen et al.(2012).

Chapter 3: Attempts at Establishing a Successful Algal Transformation Method

3.1 Introduction

Algal transformation has the potential to be a powerful tool to investigate the molecular mechanisms of proliferation and oil production. With successful transformation of algae, their ability to over produce lipids for fuel may be investigated and modulated. In addition, eukaryotic molecules beneficial to human health such as antimicrobial agents, anti-inflammatory compounds, nutrients, and other complex molecules also may be produced (Guedes et al., 2011). There are four major ways in which algal transformation can take place. Transformation can be performed through agitation, particle bombardment, poly ethylene glycol (PEG) mediated transformation, and electroporation (Appendix Table A and references therein). However, these protocols are not universally applicable across all species of algae. There have only been protocols published for about 26 algal species; 13 of them are associated with green algae, which tend to be recognized as the more promising organisms for biodiesel production as diatoms need Si supplemented media and the highest oil producers tend to be green algae (Demirbas & Demirbas, 2011). As a result, I attempted to find the most appropriate green algal species to use as a model system for basic research, as well as to produce a standard operating procedure for algal transformation.

The glass bead and silicon carbide whisker protocols are simple and easily executed, but produce low transformation efficiency in comparison to more modern protocols (Kindle, 1990, Dunahay, 1993). Due to the higher efficiency techniques such as electroporation, these agitation protocols are not used as often anymore (Brown et al., 1991, Shimogawara et al., 1998). Additionally, these protocols have only been demonstrated to work with *Chlamydomonas reinhardtii*, which has the ability to regenerate after protoplasting, a feature uncommon for most

algae. *C. reinhardtii* is not considered an ideal organism for lipid production unless working with a starch deficient mutant (Siaut et al., 2011). It is a fresh water species with the wild type strain accumulating relatively low amounts of oil and it does not perform mitotic homologous recombination easily (Gumpel *et al.*, 1994). Therefore, even though *C. reinhardtii* is one of the most common organisms used for molecular studies, it is not the best option for biodiesel production. However, it may play an important role in the future for hydrogen production (Tamburic *et al.*, 2011). As a result, *C. reinhardtii* and agitation was not investigated for this research.

Another protocol used for transformation is particle bombardment. This method may be difficult to use over long periods of time due to the financial burden it places on the researcher. For particle bombardment, gold particles are preferred, but the value of gold fluctuates and due to the current state of the economy, gold is at an all-time high of about \$1,700 oz^{-1}. Tungsten particles can also be used for transformation as a cheaper option, but they have a lower transformation efficiency compared to gold, they are more variable in size resulting in many large projectiles, and they can be toxic to cells (Randolph-Anderson et al., 1995). Moreover, while there are publications reporting transformation of microalgae by particle bombardment, it not ideal because the metal beads used as projectiles are no smaller than ~1 µm in size (Heiser, 1992). This is a problematic because the size of most microalgae ranges from 2-15 µm in diameter. As a result, even if the minimum size bead hit the maximum size alga, there is the potential to perform serious damage to the cell. Additionally, if a particle enters an alga cell, there is a high probability that the projectile will transform the plastid inside the cell; the chloroplast takes up the largest portion of the cytosol within microalgae. Thus, use of relatively large particles result in transformation of the chloroplast DNA rather than the nuclear DNA

(Doetsch et al., 2001, Lapidot et al., 2002). Finally, sterile and aseptic techniques are difficult with particle bombardment. Nevertheless, even with these limitations and the low probability of nuclear transformation, particle bombardment is not a protocol that should be completely disregarded and should be considered as a possible algal transformation procedure.

PEG mediated transformation has the potential to result in DNA uptake for algae, but most algal species have yet to be regenerated or produce viable colonies after the protoplasting event that removes the cell wall; there are no apparently viable cells (Sheehan et al., 1998). Additionally, some organisms such as *Chlorella* sp. have cell walls that contain a sporopollenin layer (Atkinson *et al.*, 1972). This is one of the strongest materials in the world and is extremely difficult to break (Atkinson et al., 1972). As a result, the concentration and intensity of enzymes needed to break down these cell walls are so excessive that the environment for the cells becomes very toxic, resulting in cell death (Atkinson et al., 1972). If protoplasting and regeneration were successful, algal transformation could potentially be performed with reasonable efficiency. Additionally, if algae were able to regenerate after protoplasting, then the agitation protocols could be reconsidered as another means of transformation.

Lastly, there is electroporation, which has produced published transformation protocols for a few species of algae, however not all protocols are practical, or valid (Table 3.1 and Appendix Table A). One of the most commonly cited methods for transforming algae uses an electroporator required a salt buffer and pulsed upwards of 2^{10} times for a single transformation, but the electroporation system it is no longer being manufactured (Chen et al., 2001). There is also a protocol that uses more reasonable parameters of a single pulse, non-salt buffer, and attainable capacitance without the need of a module, however, it has never been reproduced (Chow & Tung, 1999).

Until 2011, the only successful and reproducible electroporation protocols published involve species that are not ideal for use when producing lipids for biodiesel production (Table 3.1 and Appendix Table A). The marine algae *Nannochloropsis* sp. was the organism that changed this trend, as it was shown to be transformable as well as to have efficient mitotic homologous recombination (Kilian et al., 2011). This report describes manageable parameters, a 2.6 transformants per 10^6 cells per µg of DNA efficiency, and the ability to maintain aseptic technique with a high oil producing marine organism (Kilian et al., 2011). *Nannochloropsis* sp. is therefore a strong candidate for moving forward with algal molecular manipulation.

Attempts in this study to produce a standard operating procedure for algal transformation included PEG mediated transformation, particle bombardment, and electroporation. The organisms used for these protocols included *Coccomyxa subellipsoidea* C169, *Chlorella ellipsoidea* C211, *Chlorella vulgaris*, *Ettlia oleoabundans*, and *Nannochloropsis oceanica*. There were no successful regenerated cells after protoplasting, and only contamination occurred on recovery plates with no transformants for particle bombardment. Therefore, electroporation was investigated in more depth, but with no viable transformant.

Table 3.1:Summarization of transformation protocols for eukaryotic photosynthetic microorganisms as determined by Appendix Table A in the appendix.

Transformations	Green Algae	Diatoms	Red Algae	Protists
Agitation	2	-	-	-
Bombardment	8	9	1	-
PEG Mediated	2	-	-	-
Electroporation	11	-	2	-
Agrobacterium	1	-	1	2

3.2 Materials and Methods

Cell Culture

Coccomyxa subellipsoidea C169 a gift from Dr. James Gurnon at the University of Nebraska, *Chlorella ellipsoidea* C211, *Chlorella vulgaris*, and *Ettlia oleoabundans* were all suspended in 30 mL of BBM media (0.43 mM K_2HPO_4, 1.30 mM KH_2PO_4, 0.31 mM $MgSO_4·7H_2O$, 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2·2H_2O$, 0.43 mM NaCl, 0.17 mM EDTA – Na_4, 0.55 mM KOH, 0.018 mM $FeSO_4·7H_2O$, 0.019 mM H_2SO_4, 0.17 mM H_3BO_3, 4.91 µM $ZnSO_4·7H_2O$, 1.12 µM $MnCl_2·4H_2O$, 1.01 µM $CuSO_4·5H_2O$, 0.28 µM $Co(NO_3)_2·5H_2O$, 088 µM $Na_2MoO_4·2H_2O$) (Bold, 1949, Bischoff & Bold, 1963). *Nannochloropsis oceanica* was a gift from Dr. Christoph Benning at Michigan State University, and cultures were suspended in 30 mL of modified F/2 media (10 mM Tris pH 7.6, 24 µM $FeCl_3·6H_2O$, 15.4 µM EDTA, 2.5 mM KNO_3, 0.21 mM NaH_2PO_4, 0.20 µM $CuSO_4·5H_2O$, 0.14 µM $Na_2MoO_4·2H_2O$, 0.38 µM $ZnSO_4·7H_2O$, 0.21 µM $CoCl_2·6H_2O$, 4.54 µM $MnCl_2·4H_2O$, 20 mM $NaHCO_3$, 3.70 µM Vitamin B12 in filtered salt water) (Guillard & Ryther, 1962) with five times the amount of trace metals and KNO_3 used as a nitrogen source. Both BBM and F/2 30 mL cultures were maintained in 250 mL flasks at 125 rpm at 25°C, and exposed to 75 – 125 µmol m^{-2} s^{-1} of light from 20W GE cool white fluorescent lights (model F20T12). Cultures were periodically monitored, and growth curves were created (Figure 3.1).

Plasmid Construction

Table B in the appendix displays a list of all plasmids used across all transformation attempts. Plasmids that were not obtained from other researchers were personally created through the gateway cloning system (GCS). The Ubi 2X GFP and Ubi 6X GFP constructs were made through LR reactions of plasmid186 (pL1mEGFPR5) with plasmid201 (pL5mEGFPL2)

and plasmid190 (pL13xmEGFPR5) with plasmid192 (pL53XGFPL2) respectively. All initial plasmids (186, 201, 190, and 192) were previously prepared in entry cloning constructs by the Vidali lab with either one or three green fluorescent protein (GFP) DNA sequences. The Vidali lab pTHUbi destination vector was used to create an Ubi 2X GFP and Ubi 6X GFP plasmid with the GFPs driven by an ubiquitin promoter. Additionally, the pTHUbi vector contains a hygromycin resistance gene with a mosaic virus 35S promoter.

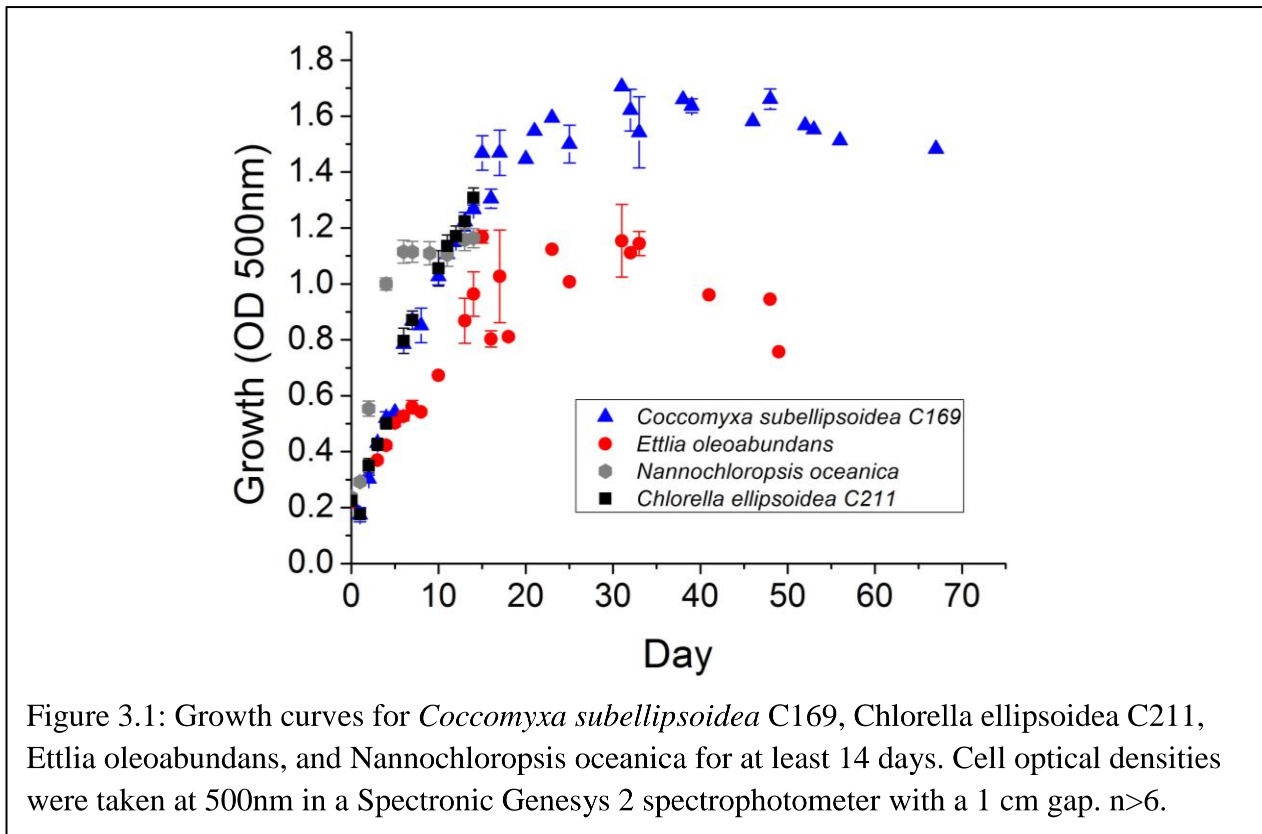

Figure 3.1: Growth curves for *Coccomyxa subellipsoidea* C169, Chlorella ellipsoidea C211, Ettlia oleoabundans, and Nannochloropsis oceanica for at least 14 days. Cell optical densities were taken at 500nm in a Spectronic Genesys 2 spectrophotometer with a 1 cm gap. n>6.

The yellow fluorescent protein (YFP) sequence used for all YFP plasmids was obtained through PCR from the *Shizosaccharomyces pombe* strain JW1041. This strain was donated to the Vidali lab from Dr. Thomas Pollard and contains a monomeric YFP (Wu & Pollard, 2005). JW1041 was reconstituted on a YEPD agar plate for 3 days. A sample was then inoculated into a 5 ml culture of YEPD and grown overnight, and DNA was collected using the smash and grab lab protocol from the Prusty Rao lab. The culture was pelleted at 11,000 X g for 2 minutes, the

supernatant discarded, and the culture resuspended into 200μL S&G buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA mL^{-1}). To the suspension 200 μL phenol chloroform was added and 0.3 g of 425-600 μm glass beads (Sigma G8772). The mixture was vortexed for 5 minutes and then centrifuged for 5 minutes at 11,000 X g. A 200 μL aliquot of supernatant was then added to a new tube containing 20 μL of 3 M sodium acetate and 500 μL 100% ethanol. The mixture was placed at -20°C for 10 minutes and then centrifuged again for 2 minutes at 11,000 X g. The supernatant was discarded and the pellet was air dried for 5-10 minutes and 50 μL of sterile DI H_2O was used to resuspend the DNA pellet.

A PCR was run to amplify the YFP sequence from the yeast DNA. With the amplified and purified sequence acquired, a BP reaction of the PCR fragment, added the DNA into the entry clone. An LR reaction was used afterwards to insert the fragment into three different destination vectors. All three vectors contained a hygromycin resistance cassette, which is driven by a mosaic virus 35S promoter, while the YFP sequence in each vector is driven by either an ubiquitin promoter, a single 35S promoter, or a double 35S promoter.

PEG Mediated Transformation

PEG mediated transformation was performed as described in Liu and Vidali (Liu & Vidali, 2011). The only modifications to the protocol were that the alga cells were protoplasted for 24 hours with a 1% driselase (Sigma D8037), 2% cellulose (Shin Nihon Chemical Co.), and 2% pectinase (Shin Nihon Chemical Co.) mixture, and 6×10^6 cells mL^{-1}.

Protoplast experiments

Cells were protoplasted using 24 hour reactions. A sample of cells in mid log phase was centrifuged at 1700 rpm (700 x g) for 15 minutes in an IEC Centra-7 centrifuge with a swinging bucket rotor. The pellet was resuspended in 10 – 30mL of a protoplasting enzyme solution

containing a mixture of driselase (Sigma D8037), macerase (Calbiochem 441201), Macerozyme (MP Biomedicals, LLC 152340), cellulose (Shin Nihon Chemical Co.), and or pectinase (Shin Nihon Chemical Co.) (Table 3.2). All enzymes were diluted in 0.3 M sorbitol and 0.3 M mannitol or 3.075 mM Na_2HPO_4, 21.925 mM NaH_2PO_4, 0.3 M sorbitol and 0.3 M mannitol.

Table 3.2: List and key of protoplast mixtures used for experiments in percent (w v^{-1}).

Enzyme Code	Driselase	Cellulase	Pectinase	Macerase	Macerozyme
D	2%	-	-	-	-
DCP	1%	2%	2%	-	-
CP	-	4%	4%	-	-
CPM	-	4%	1%	2%	-
CPMz		4%	2%	0.75%	
DCPM	1%	2%	0.5%	1%	-

Electroporation

Specific electroporations can be seen in Table 3.5. All electroporations consisted of collecting log phase cultures (Figure 3.1) of algae 700g for 15 minute with a swinging bucket rotter. Wash three times in a high osmoticum such as 0.2 M mannitol and 0.2 M sorbitol, 8% mannitol, or 375 mM sorbitol and centrifuging at 700g for 15 minutes in a swinging bucket rotter. After the final wash, resuspend in the final buffer intended for electroporation. Early attempts at electroporation consisted of using a high salt electroporation buffer, which was diluted 1:10 in sterile water before resuspending the samples intended for electroporation (Chen et al., 2001). This was done in order to reduce that amount of arcing that took place with the full salt concentration. Other buffers used for electroporation did not contain any salts in order to keep the resistance low. These new buffers consisted of 0.2 M mannitol and 0.2 M sorbitol, TAP media with 20% sucrose, sterile water, or 375 mM sorbitol. The final cell densities ranged from $1x10^6$ to $2.5x10^8$ cells mL^{-1} and transformation attempt parameters ranged from 0.2 KV to 3.0 KV (1KV-15KV cm^{-1}), 10 µF-40µF, 400 Ω-1000 Ω with the Bio-Rad MicroPulser, Bio-Rad

Gene Pulser I, BTX ECM600, and the Bio-Rad Xcell Electroporation system with a PC module. Parameters are listed in Table 3.5.

After electroporation, all algal cells were recovered with 1 mL of liquid media from the electroporation cuvette. In initial tests, cells were plated directly onto media agar and then switched to hygromycin agar for selection. Later procedures, cells were allowed to recover for 1-2 days in 5mL media agar until they were plated with top agar onto hygromycin selection plates.

3.4 Results and Discussion

As a starting point, and before the publication of the *Nannochloropsis* sp. transformation protocol, experiments were designed towards transforming *C. subellipsoidea* C169, *E. oleoabundans*, *C. ellipsoidea* C211, and *C. vulgaris* (Kilian et al., 2011). In order to determine if transformation took place, plasmids containing the selectable marker hygromycin and fluorescently tagged proteins were constructed or obtained from other investigators (Appendix Table B). GFP expression was initially selected to screen for successful transformation using fluorescence, however using the 470 nm filter set gave background auto-fluorescence from dead cells making use of GFP fluorescence difficult to discern between dead cells or fluorescence from the expressed protein. YFP constructs were subsequently made and further used to screen for transformants due to a lack of background fluorescence from dead cells within the YFP 497nm filter set.

Electroporation and PEG mediated transformations were the two major methods tested for this project. Glass bead transformation was not attempted due to its ability to only work with *C. reinhardtii* and because of the necessary removal of the cell wall followed by regeneration. Gold particle bombardment was attempted once, but produced a large amount of contamination and a lack of positive results.

The first attempt at PEG mediated transformation for the algae used the Liu and Vidali (2011) transformation protocol in conjunction with the Ubi-SuSy plasmid (Appendix Table B), which expresses the soluble protein sucrose-synthase fused to GFP. Initial attempts at PEG mediated transformation produced no results of plasmid driven fluorescence, nor was there a strong indication of hygromycin resistance as over time all the cells died. The lack of viable cells led to a further investigation of the effect of protoplasting in cell viability. A variety of enzymes and green algae combinations were tested to find a protocol that would result in the removal of cell wall and subsequent regeneration (Table 3.3). As more attempts to protoplast these cells were performed, it became clear that most of the enzymes tested for cell wall removal did not readily weaken or digest the algal cell wall. This is most likely due to the sporopollenin layer found on the outside of the cells walls of some algae, such as *Chlorella* species (Atkinson et al., 1972). In fact, most cells were observed to continue dividing and their numbers grew into the next day after the start of the 24 hour enzymatic treatment (Table 3.4). The only species that was readily protoplasted was *Ettlia oleoabundans*. However, consistent with previous algae protoplasting attempts, *E. oleoabundans* was unable to survive the treatment, as shown in Table 3.4 (Sheehan et al., 1998). The control cells, where enzyme was omitted from the treatment, did survive and displayed a healthy lawn of growth, however, even after the initial introduction of the enzyme, more than half of the culture was no longer viable. Even within two hours of treatment, living cells that remained left could only be seen under the microscope due to their chlorophyll autofluorescence, but did not proliferate into macroscopic visible growth. After 7 hours, no more viable cells were observed; this was only a third of the usual time recommended for protoplasting (Yamada & Sakaguchi, 1982, Yamada & Sakaguchi, 1981, Aach et al., 1978).

As a result, PEG mediated transformation, which requires protoplasting and regeneration, was not considered practical and was not further investigated.

Table 3.3: Averaged protoplasting results for *E. oleoabundans*, *C. ellipsoidea* C211, and *C. subellipsoidea* C169. Enzyme mixtures D, CP, DCP, CPM, CPMz, and DMCP correspond to Table 3.2. DNP = Did Not Protoplast.

Organism	D	CP	DCP	CPM	CPMz	DCPM
Ettlia oleoabundans	DNP	DNP	30.63%	29.01%	23.95	DNP
Chlorella ellipsoidea C211	DNP	DNP	DNP	DNP	DNP	DNP
Coccomyxa sp. C169	DNP	DNP	DNP	DNP	DNP	DNP

Without the option of protoplasting and the impracticality of particle bombardment, electroporation was the only feasible option left to explore. Multiple transformation attempts were performed using three different electroporators: the Bio-Rad MicroPulser, the Bio-Rad Gene Pulser I - BTX ECM600, and the Bio-Rad Xcell Electroporation system with a PC module. Parameters ranged from 0.2 KV to 3.0 KV (1 KV-15 KV cm^{-1}), 10 µF to 40 µF, and 400 Ω to 1000 Ω, with either *C. subellipsoidea* C169, *C. ellipsoidea* C211, or *C. vulgaris* (Table 3.5). Both linearized and circular plasmids were used and neither made a difference in the results obtained. Overall, no transformants were obtained, however, at a very low frequency hygromycin resistant colonies of *Chlorella vulgaris* were found after attempting the electro-transformation protocol (Table 3.5 No. 8) (Chow & Tung, 1999). Every viable colony was saved and continued to grow both on hygromycin BBM agar and in hygromycin BBM liquid media. When the resistant colonies were investigated further it was discovered through PCR, that the original stock of *C. vulgaris*, contains a similar sequence to the gene that codes for hygromycin resistance, and PCR products primed for sections of the plasmid backbone could not be amplified (Figure 3.2 and 3.3). Therefore, it was accepted that the plasmid was not transformed in to the cells and the resistance had developed within a small population of the WT culture due to a spontaneous mutation.

Table 3.4: *Ettlia oleoabundans* hourly protoplasting results.

Hours	% Protoplasts	Colonies	Viable Cells
0	12.50%	Lawn	Yes
1	12.00%	Yes	Yes
2	1.86%	No	Yes
4	-7.57%	No	Yes
5	16.53%	No	Yes
6	18.60%	No	Yes
7	10.84%	No	Yes
8	22.64%	No	No
24	5.61%	No	No

A recent publication reported that *Nannochloropsis* sp. could be transformed via electroporation with a transformation efficiency of 2.6 transformants per 10^6 cells per µg of DNA, and this alga could perform mitotic homologous recombination (Kilian et al., 2011). Mitotic homologous recombination is a powerful tool that can be used to explore gene function and for metabolic engineering, both critical tools are required to further develop our knowledge and applications of oil producing algae. Consequently, the reported strains and transformation plasmids were obtained and transformation were performed attempts using the protocol cited (Table 3.5 No. 12). The company that published the report, Aurora Algae, provided both the *Nannochloropsis* sp. Culture and the VCP1::H8 plasmid (Appendix Table B) containing the hygromycin resistance cassette. Despite, closely following published protocols, including the addition of a Bio-Rad Pulse Controller PC module to the Bio-Rad Xcell electroporation system, no transformed colonies were produced. The only variable left to alter in order to obtain transformed *N. oceanica* colonies would be to use a younger starting culture as it was later determined that 1 week old cultures of *N. oceanica* are already in stationary phase (Figure 3.1).

Table 3.5: Algal transformation attempts through electroporation. Described in the table are the organism used, the system it was used in, the plasmid and parameters, as well as the results. All experiments were performed with a 0.2cm gap Bio-Rad Gene Pulser Cuvette. HR – Hygromycin, F – Fluorescence. lin – linearized, and if unspecified, the plasmid was circular.

No.	Organism	Electroporator	Plasmid	Vol(μL)/V(kV)/μF/Ω/Pulses	HR	F
1	*Coccomyxa* sp. C169	Bio-Rad micropulser	Ubi::susy	200 / 1.5 / 10 / 600 / 1-3	No	No
2	*Coccomyxa* sp. C169	24hr DCP Bio-Rad micropulser	Ubi::2xGFP Ubi::6xGFP	200 / 1.5 / 10 / 600 / 1-8 200 / 2.0 / 10 / 600 / 1-8	No	No
3	*Coccomyxa* sp. C169	Bio-Rad micropulser	2x35S::mYFP, 35S::mYFP, Ubi::mYFP	50 / 1 /10 / 600 / 1	No	No
4	*Coccomyxa* sp. C169	Bio-Rad micropulser	Ubi::mYFP	50 /3 / 10 / 600 / 32	No	No
5	*Chlorella Vulgaris*	Bio-Rad micropulser	35S::mYFP	80 / 1.8 / 10 / 600 / 1	No	No
6	*Chlorella Vulgaris*	Bio-Rad micropulser	35S::mYFP	80 / 1.8 / 10 / 600 / 1	No	No
7	*Chlorella Vulgaris*	BTX ECM600	βTP::Hygro	100 / 0.2 / 1000 / 13 / 1	No	No
8	*Chlorella Vulgaris*	BTX ECM600	βTP::Hygro lin βTP::Hygro 35S::mYFP	100 / 0.2 / 1000 / 13 / 1 100 / 1 / 40 / 186 / 1 100 / 2 / 40 / 186 / 1 100 / 1.5 / 40 / 129 / 1 100 / 1.5 / 40 / 13 / 1	Yes	No
9	*Nannochloropsis oceanica*	Bio-Rad Gene Pulser I	Ubi::mYFP, lin Ubi::mYFP	2.2 / 25 / 600 / 1	No	No
10	*Nannochloropsis oceanica*	Bio-Rad Gene Pulser I	lin Ubi::mYFP	200 / 2.2 / 25 / 600 / 1 200 / 2.2 / 25 / 800 / 1 200 / 2.2 / 25 / 1000 / 1	No	No
11	*Nannochloropsis oceanica*	BTX ECM600	lin Ubi::mYFP	200 / 2.2 / 40 / 480 / 1 200 / 2.2 / 40 / 720 / 1	No	No
12	*Nannochloropsis oceanica*	Bio-Rad XCell w/ PC Module	lin Ubi::mYFP 35S::mYFP lin VCP1::H8	200 / 2.2 / 50 / 500 / 1	No	No

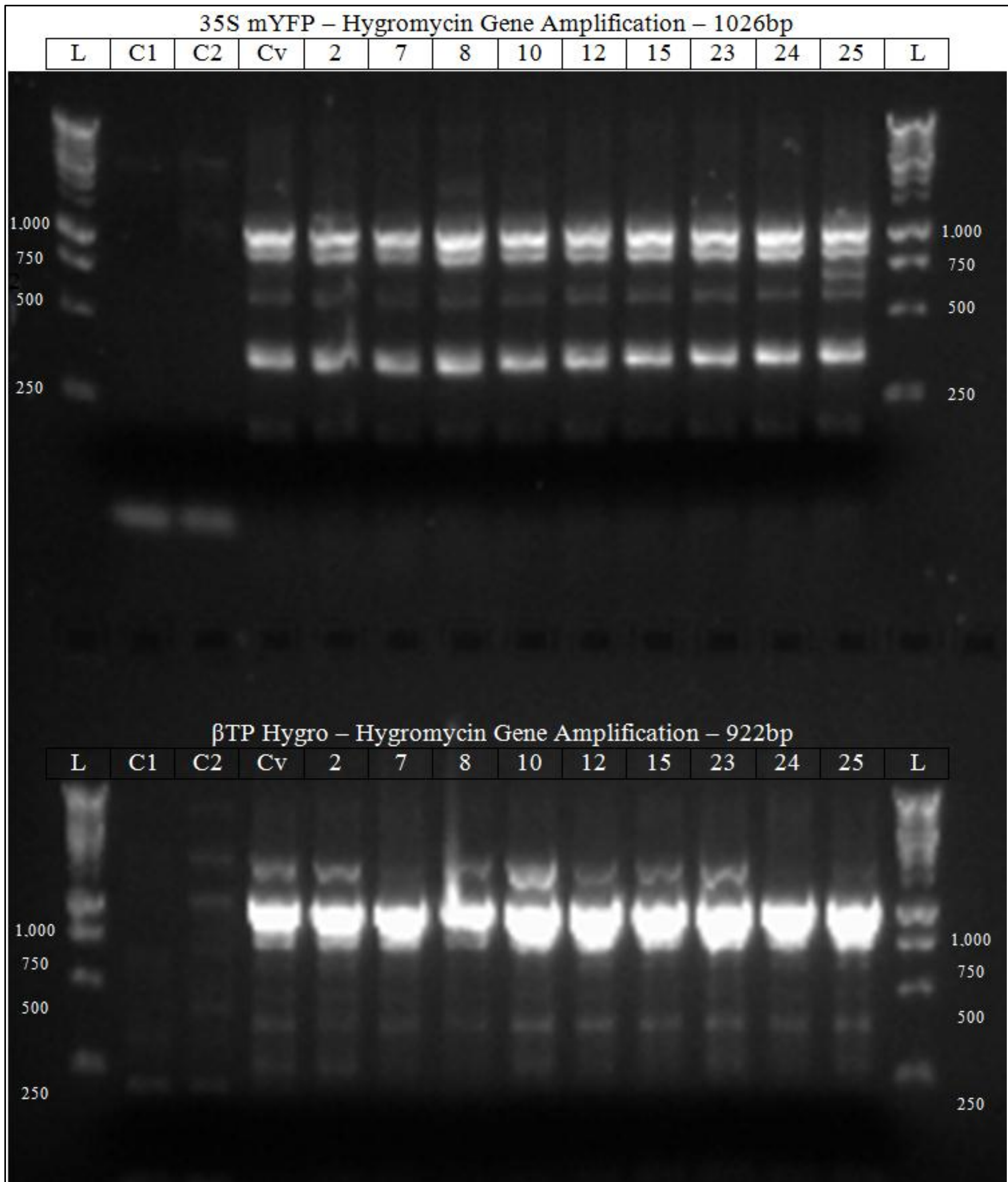

Figure 3.2: PCR amplification of 35S mYFP and βTP Hygro hygromycin resistance genes from hygromycin resistant *Chlorella vulgaris*. L – 1kb benchtop ladder, C1 – PCR water control, C2 – TE control, Cv – WT *Chlorella vulgaris* genomic DNA, 2-25 – Electroporated hygromycin resistant *Chlorella vulgaris* DNA shocked with either 35S mYFP or βTP Hygro

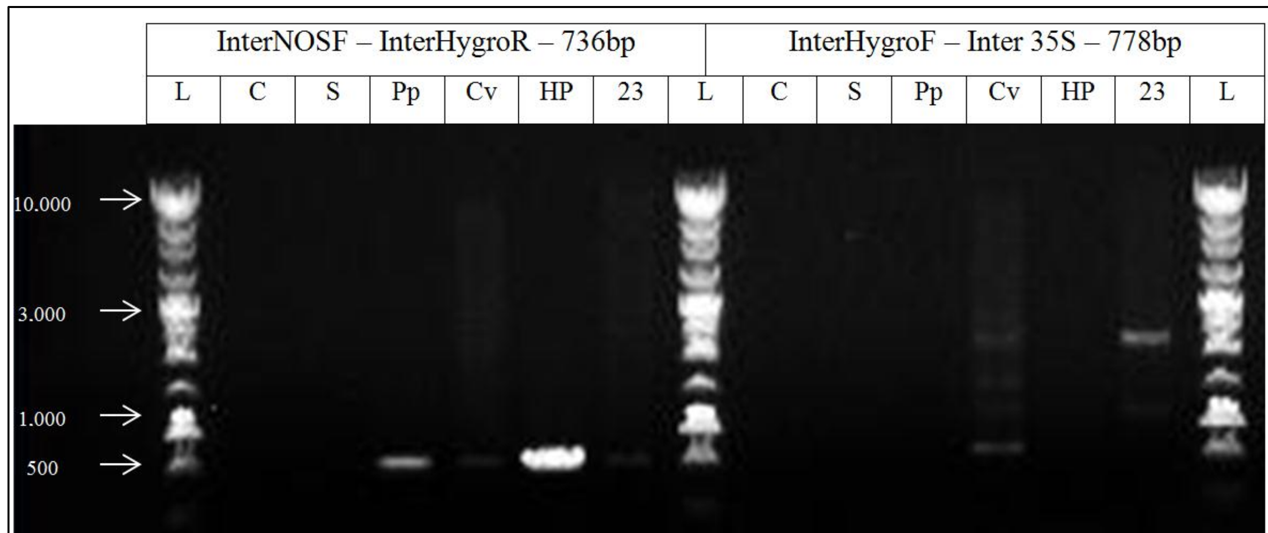

Figure 3.3: PCR amplification of hygromycin resistance plasmid 35S mYFP from hygromycin resistant *Chlorella vulgaris* transformation attempt at 200ul 1.5KV 129Ω and 40µF with the BTX ECM600. Three PCR sets were attempted to be amplified including NOS to mid hygromycin resistant gene, mid hygromycin resistance gene to mid 35S promoter, and the hygromycin resistance gene. L – 1kb benchtop ladder, C – PCR water control, S – Shorty prep control, Pp – WT gran *Physcomitrella patens* DNA, Cv – WT *Chlorella vulgaris* genomic DNA HP – Hygromycin resistant *P. patens* with a 35S driven plasmid, 23 – Hygromycin resistant C. vulgaris from transformation attempt.

3.5 Conclusion

This research summarizes the difficulties and hardships associated with eukaryotic algal transformation as few reliable protocols have been published and no transformants resulted from these experiments (Appendix Table A). The small size and thick cell walls of these organisms led to complications with using apparently established protocols for transformation. For example, removal of the cell wall leads to an inability to maintain and regenerate viable cells. Particle bombardment is difficult due to the small size of the algae and the relative large size of the projectiles. The general ruggedness of the cell wall results in cells that are not competent even with high voltage and high capacitance electroporation. Although, the published protocol with *Nannochloropsis* sp. seems promising, I could not replicate their results (Kilian et al., 2011).

Chapter 4: Rapid Solid Media-Based Microgrowth Assay for Microalgae

4.1 Introduction

Microalgae are becoming increasingly important as they have the ability to produce oil, complex compounds that are beneficial to human health, and they can be used in toxicity assays indicating potential harmful effects of a compound or material (Guedes et al., 2011, Griffiths & Harrison, 2009, Kviderova, 2010). With the increased interest in microalgae, a simple microgrowth assay could provide further insight into the molecular and cellular mechanisms that impact algal growth, cellular dynamics, and product output. Therefore, a small scale microgrowth assay that can be automated and used for a variety of microalgae would greatly benefit research by saving time, space, and supplies.

Recently, a micro toxicity assay was developed for determining half maximal inhibitory concentrations of toxins for *Chlorella kessleri* (Kviderova, 2010). However, this procedure has limitations. First, the cells were grown in liquid media using a 96 well plate; this results in problems of evaporation and agitation. Second, the plate was sealed using Parafilm to decrease media evaporation. Consequently, the plate had to be flooded with carbon dioxide to aid algal growth. This closed system complicates experimental reproducibility. Finally, the assay was used to establish a dose response by measuring chlorophyll A fluorescence, which has been shown to be indicative of algal health and toxicity of the environment (Judy *et al.*, 1991, Mecozzi & Oteri, 2011); however, simple light scattering and absorption by chlorophyll are indirect measurements of culture growth and without agitation the culture will settle within the well and create a mixture that is not homogeneous. Therefore, an assay prepared in a 96 well plate that does not have the limitations of evaporation, agitation, and indirect growth monitoring could simplify measurement of growth, increase reproducibility, and improve high throughput dose response analysis.

Here I demonstrate the effectiveness of a rapid solid media-based microassay. I measured growth yield with the nutrients NaCl, $NaNO_3$, and KPO_4 as variables, and also measured the dose response to the actin cytoskeleton inhibitory compound, latrunculin B. Using a 96 well plate and agar media, micro-colonies were imaged with fluorescence microscopy to directly quantify cell growth. By using agar to reduce evaporation, there was no need to seal the plate, and it eliminates the need for agitation. Additionally, imaging individual cells as they grow into micro colonies allows one to quantify growth directly based on colony area. Multiple conditions can be tested across the wells of the plate and the results used to determine growth effects from different concentrations of nutrients and from toxins within solid media.

4.2 Materials and Methods

Species, Culture Maintenance, and Monitoring Growth

Coccomyxa subellipsoidea C169 (*Chlorella vulgaris* C-169, Chlorophyta) was a gift from Dr. James Gurnon at the University of Nebraska. Cultures were suspended in 30 mL of BBM media (0.43 mM K_2HPO_4, 1.30 mM KH_2PO_4, 0.31 mM $MgSO_4 \cdot 7H_2O$, 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2 \cdot 2H_2O$, 0.43 mM NaCl, 0.17 mM EDTA – Na_4, 0.55 mM KOH, 0.018 mM $FeSO_4 \cdot 7H_2O$, 0.019 mM H_2SO_4, 0.17 mM H_3BO_3, 4.91 µM $ZnSO_4 \cdot 7H_2O$, 1.12 µM $MnCl_2 \cdot 4H_2O$, 1.01 µM $CuSO_4 \cdot 5H_2O$, 0.28 µM $Co(NO_3)_2 \cdot 5H_2O$, 088 µM $Na_2MoO_4 \cdot 2H_2O$) (Bold, 1949, Bischoff & Bold, 1963). *Nannochloropsis oceanica* was a gift from Dr. Christoph Benning at Michigan State University. Cultures were suspended in 30 mL of modified F/2 media (10 mM Tris pH 7.6, 24 µM $FeCl_3 \cdot 6H_2O$, 15.4 µM EDTA, 2.5 mM KNO_3, 0.21 mM NaH_2PO_4, 0.20 µM $CuSO_4 \cdot 5H_2O$, 0.14 µM $Na_2MoO_4 \cdot 2H_2O$, 0.38 µM $ZnSO_4 \cdot 7H_2O$, 0.21 µM $CoCl_2 \cdot 6H_2O$, 4.54 µM $MnCl_2 \cdot 4H_2O$, 20 mM $NaHCO_3$, 3.70 µM Vitamin B12 in filtered salt water) with five times the amount of trace metals and KNO_3 used as a nitrogen source (Guillard & Ryther, 1962).

Both cultures were maintained in 30 mL volumes in 250 mL flasks at 125 rpm at 25°C, and exposed to 75 – 125 μmol m^{-2} s^{-1} of light from 20W GE cool white fluorescent lights (model F20T12).

Microassay

Culture growth in the microassay was determined by filling all the wells of a Cellstar 96 well plate (No.655180) with 400 μL of 1% agar media using a Rainin AutoRep E repeater pipette. A 5 μL aliquot of cells was placed on top of the solidified media for the inner 60 wells again with a Rainin AutoRep E repeater pipette, and plates were placed within a Percival incubator (Model CU-36L6) with 125 μmol m^{-2} s^{-1} of cool white light (model F17T8) exposure at 25°C. The cell densities ranged from 32,000 cells $well^{-1}$ to 63 cells $well^{-1}$. The perimeter wells were not used for cellular growth because there was minor evaporation along the perimeter of the plate (Figure 4.1 A and B).

Over a period of 1 week, pictures of microcolonies were taken using a Zeiss AxioCam MRc camera with the Axio Vision Release 4.6 imaging software on a Zeiss SteREO Discovery.V12 microscope with a fluorescence cube with a 480/40 bandpass excitation filter, a 505 longpass dichroic mirror, and a 510 longpass emission filter. The individual microcolonies of the inner sixty wells were imaged while exciting the chlorophyll at 480nm using a 10X lens and 32.5X zoom (Figure 4.1 C and D).

NaCl, $NaNO_3$, and KPO_4 were chosen as nutrients to test *C. subellipsoidea* C169 growth. All stock solutions were made using deionized water with the NaCl stock concentration at 1.37 M and the $NaNO_3$ stock concentration at 2.94 M. The KPO_4 stock solution was designed with a 1:1 ratio of K_2HPO_4 (0.43 M) and KH_2PO_4 (1.30 M) with a 1.73 M concentration of PO_4. Into the inner 60 wells, 4 μL of the appropriate variable nutrient were aliquoted creating a high to low

nutrient concentration range across the plate with each column representing a specific concentration. To create the high to low concentration range, the stocks were serial diluted by half for experimental columns 2-9 on the plate and the 10th column contained sterile water instead of the variable nutrient. 1% agar BBM lacking the variable component was then dispensed into every well using a Rainin repeater pipette for a final volume of 400 μL, diluting the variable nutrient within the inner 60 wells 1:100 (v v^{-1}). The perimeter wells were also filled with media agar; however, they were not used for cellular growth. A log phase sample of *C. subellipsoidea* C169 was diluted with sterile water to 200 cells μL^{-1}. The algae were plated in 5 μL aliquots on surface of agar in the inner 60 experimental wells for a final concentration of 1,000 cells $well^{-1}$. Plates were incubated in the Percival incubator and pictures were taken as described.

To determine the dose response of *N. oceanica* to a growth inhibitor, the actin depolymerizing inhibitor latrunculin B was used; it was dissolved in 100% ethanol to. This initial stock of 20 mM was then diluted 1:10 v v^{-1} in sterile H_2O resulting in a 10% ethanol solution. Using 10% ethanol, the working solutions of the drug were then serial diluted by half for experimental columns 2-8 of the plate. Latrunculin B was dispensed in 2 μL aliquots into each of the inner 60 wells of the 96 well plates. The final two columns were a 10% ethanol control with no drug and a sterile H_2O control. An aliquot of 1% modified F/2 agar was then dispensed into every well to a final volume of 400 μL, resulting in a 1:200 dilution v v^{-1} of the inner 60 experimental wells. A log phase *N. oceanica* culture was diluted to 50 cells μL^{-1} in liquid modified F/2 and plated in 5 μL aliquots across the plate resulting at a concentration of 250 cells $well^{-1}$. Plates were incubated in the Percival incubator and pictures were taken as described.

Liquid Culture Growth with Variable Nutrients

The liquid growth assay was performed with log phase *C. subellipsoidea* C169 cells that were washed 3 times with sterile water. The cells were centrifuged for 10 minutes at 400 x g and then resuspended into BBM lacking the test nutrient. The test nutrient was added to the appropriate 50 mL Pyrex glass test tubes (No. 9820-25) and diluted 1:100 ($v\ v^{-1}$) with the suspension of cells with a starting OD of 0.05 cm^{-1} at 500nm and a final volume of 10 mL. Over a period of 1 week, the culture tubes were placed on a slant rack and agitated at 100 rpm with 115-130 µmol $m^{-2}\ s^{-1}$ light from 15W GE cool white light fluorescent tube bulbs (model F15T8-CW). Culture density was measured in situ using a Thermo Spectronic 20D+ with a 1:5 dilution ($v\ v^{-1}$) made on day 7 to obtain an OD reading at 500 nm.

Data and Statistical Analyses

Microassay pictures were analyzed using ImageJ (NIH, 2004) and an in-house developed macro. The macro analyzes each set of pictures, collecting the data of every micro colony in the field of view. The macro processes the images by deleting the green and blue channels leaving only the red channel, applying a Gaussian blur filter on the image in order to smooth the edges of the colonies, and then determining colony area with an automatic threshold function, set to quantify only the full area of each colony in the field of view that does not touch the image's edge. Only occasionally, the threshold that determines the colony size must be adjusted manually in order to ensure that the macro quantifies the entire colony area. The macro is available upon request.

OriginPro 8.1 (Origin Lab Corporation) was used for statistical analysis of the colony size and optical density data, generating averages and standard errors. Each microassay plate and set of liquid culture tubes counted as $n = 1$, but for each microassay a total of 6 to 132 colonies

were measured per variable concentration. Therefore, each n was the average area of all the colonies imaged in a specific column from a plate, corresponding to a single value at a specific nutrient concentration. Every plate column value was then averaged together and reported as a single average with error bars displaying standard error between plates from independent experiments. Statistical significance for the dose response assays was determined by comparing the raw colony size for a specific condition through a single factor ANOVA test within OriginPro.

4.3 Results and Discussion

Coccomyxa subellipsoidea C169 (*Chlorella vulgaris* C169, Chlorophyta) and *Nannochloropsis oceanica* were chosen to develop this assay because their non-motile, simple structure, single celled morphology, a completed genome sequence, and the potential to be molecularly manipulated (JGI, 2009, Pan et al., 2011, Yamada & Sakaguchi, 1982, Chow & Tung, 1999, Hawkins & Nakamura, 1999, Kilian et al., 2011). *C. subellipsoidea* C169 and *N. oceanica* are also oil producing algae with the potential to provide insight into algal cell growth and proliferation, critical aspects for the optimization of oil production in microalgae (Msanne *et al.*, 2012, Rodolfi *et al.*, 2009).

To determine the cell density that provides single micro colonies and avoids confluence, *C. subellipsoidea* C169 was grown in 96-well plates for seven days. It was determined that 1000 cells per well was the appropriate sample size to image single micro colonies from single cells (Figure 4.1 C right panels). Cell numbers higher than 1,000 cells per well displayed excessive merging, confluence, and a lack of discernible colonies, making analysis difficult and unreliable when trying to measure single microcolonies as displayed at 32,000 cells well^{-1} (Figure 4.1 C left panels). With the appropriate cell number determined, NaCl, $NaNO_3$, and KPO_4 nutrients were

tested to determine concentrations that produce maximum growth for *C. subellipsoidea* C169. For comparison, a series of 10 mL liquid growth cultures were run in parallel with the microassay.

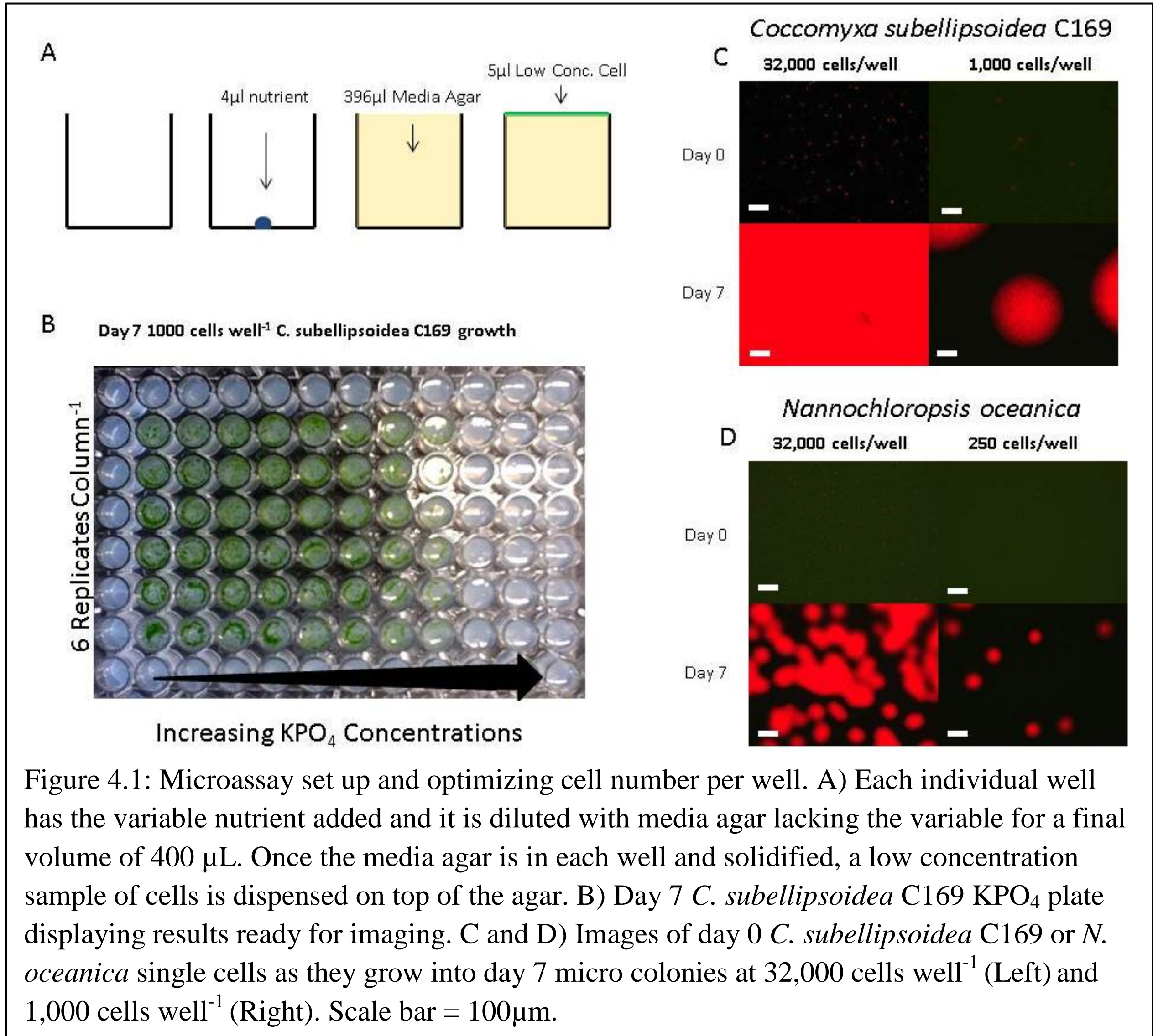

Figure 4.1: Microassay set up and optimizing cell number per well. A) Each individual well has the variable nutrient added and it is diluted with media agar lacking the variable for a final volume of 400 µL. Once the media agar is in each well and solidified, a low concentration sample of cells is dispensed on top of the agar. B) Day 7 *C. subellipsoidea* C169 KPO_4 plate displaying results ready for imaging. C and D) Images of day 0 *C. subellipsoidea* C169 or *N. oceanica* single cells as they grow into day 7 micro colonies at 32,000 cells $well^{-1}$ (Left) and 1,000 cells $well^{-1}$ (Right). Scale bar = 100µm.

When NaCl was the variable nutrient in the microassay, only low concentrations were tested due to the fresh water nature of *C. subellipsoidea* C169. However, our goal was to determine if a greater level of salinity could be tolerated when the algae were grown on solid media. The concentration range used was 0 - 13.7 mM NaCl; the latter is slightly above

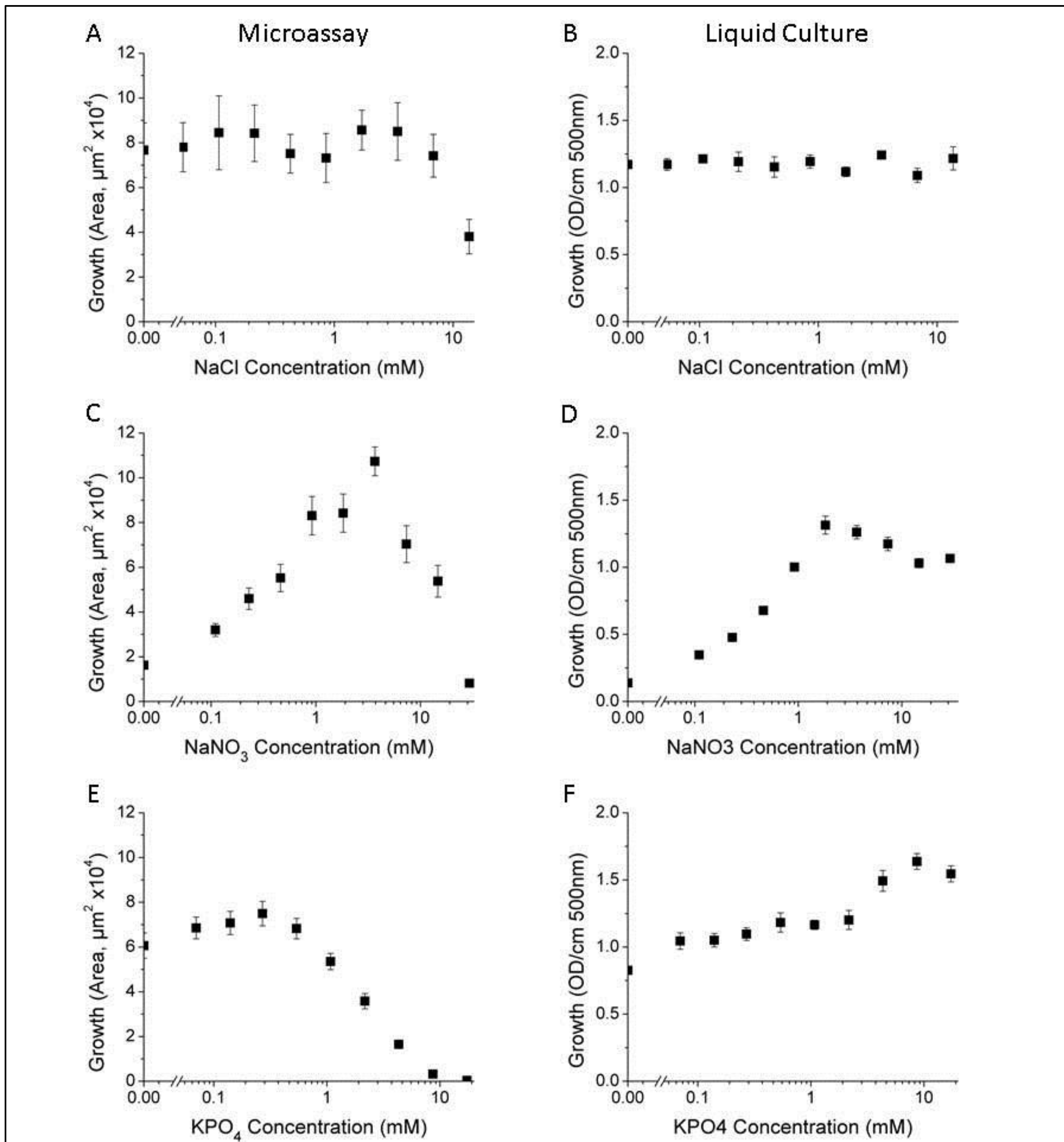

Figure 4.2: Day 7 *C. subellipsoidea* C169 results for the microassay (Left) and liquid culture (Right) results for NaCl (A and B), $NaNO_3$ (C and D), and KPO_4 (E and F) with BBM as the base media. The gradient of NaCl created was 0.00 mM 0.05 mM, 0.11 mM, 0.21 mM, 0.43 mM, 0.86 mM, 1.71 mM, 3.43 mM, 6.85 mM, 13.7 mM. The gradient of NaNO3 created was 0.00 mM, 0.11 mM, 0.23 mM, 0.46 mM, 0.92 mM, 1.84 mM, 3.68 mM, 7.35 mM, 14.7 mM, 29.4 mM. The gradient of KPO_4 created was 0.00 mM, 0.07 mM, 0.14 mM, 0.27 mM, 0.54 mM, 1.08 mM, 2.17 mM, 4.33 mM, 8.67 mM, 17.34 mM. Error bars represent standard error, A n=4, B n=3, C n=5, D n=4, E n=4, F n=7.

freshwater salinity (<8.56 mM NaCl). The results from the microassay showed that there does not appear to be any particular optimal NaCl concentration for maximum alga growth (Figure 4.2 A). The microassay also showed cellular toxicity as there was minimal growth in wells containing 13.7 mM NaCl (Figure 4.2 A). When compared to growth in liquid culture, the overall trends between the two culture systems are similar with the exception that the liquid cultures did not show toxicity at 13.7 mM NaCl (Figure 4.2 A and B). This observation highlights that under solid growth conditions the algae adopt different physiological states that may be critical for colony growth. Additionally, the agreement between the microassay and the liquid culture suggests that NaCl may not be necessary in BBM media for *C. subellipsoidea* C169.

The concentrations of $NaNO_3$ tested in the microassay ranged from 0 - 29.4 mM. The concentration that produced the maximum growth from the microassay was 3.68 mM $NaNO_3$, and at the highest nitrate concentration of 29.4 mM toxicity was displayed; there was little to no growth (Figure 4.2 C). When viewing the liquid culture, a maximum growth yield in liquid was observed with a 1.84mM $NaNO_3$ BBM concentration, minimal growth was seen at low nitrate concentrations, and it was noted that there is slight toxicity at the high nitrate concentrations (Figure 4.2 D). These results obtained with increasing levels of $NaNO_3$ demonstrate that the microgrowth assay is a sensitive method for determining maximum growth and inhibitory concentrations in response to variable nitrate concentrations.

When determining the optimal KPO_4 concentration using the microassay, the range of KPO_4 used was 0 - 17.34 mM. It was observed that the two highest concentrations tested, 17.34 mM and 8.67 mM, produced little to no growth and the maximum growth was at 0.27 mM KPO_4 (Figure 4.2 E). The optimal microassay KPO_4 concentration contrasts considerably with the

optimal liquid medium concentration of 8.67mM (Figure 4.2 F). For comparison, the BBM standard medium is 1.73 mM KPO_4. Interestingly, as seen before with NaCl, at very low levels of KPO_4 there is no major effect on growth (Figure 4.2 E). However, the contrast between the microassay and liquid culture results suggest that phosphate use, storage, and or assimilation between solid and liquid media affect algal growth differently between the two assays (Figure 4.2 E and F). Understanding the underlying physiological adaptations that allow this differential response may provide clues about how to reduce phosphate use for large-scale algal production systems.

To investigate the discrepancy in phosphate use between the liquid and agar media, phosphate concentrations in the liquid culture medium were measured. The difference between the initial and final phosphate concentrations at each concentration suggested that the algae may indeed be recycling phosphate between cells and their medium. Many algae conserve their phosphate via formation of polyphosphate bodies, so unless other nutrients are available, e.g. nitrate and CO_2, growth ceases (Powell *et al.*, 2009). Additionally, it is interesting that the 1.73mM KPO_4 concentration dictated by the standard BBM recipe appears to provide good growth for both solid and liquid media (Figure 4.2 E and F).

To investigate the use of the current protocol as a toxicity microassay and to show that the current growth assay can be used with other algal species, *N. oceanica* was used instead of C169, but following the same analytical method. It was established that 250 cells well^{-1} was the appropriate concentration (Figure 4.1 D). Cell concentrations above 250 cells well^{-1} displayed excessive merging and confluence as shown at 32,000 cells well^{-1} (Figure 4.1 D left panel). The drug used for this assay was the actin depolymerizing agent, latrunculin B. The range tested was from 0 μM to a maximal concentration of 10 μM. With the results normalized to the 0.05 %

ethanol control there was a strong dose response with a half maximal inhibitory concentration at 2.90 µM (Figure 4.3). A statistical two sample T-Test analysis between the 0.05% ethanol and sterile water controls showed there was no significant difference (n=3). The microassay, therefore, shows that an EC50 due to drug toxicity can be determined on solid media without the limitations of using miniaturized liquid cultures (Kviderova, 2010). Many valuable drugs that inhibit important cellular processes are expensive, so this method conserves material while allowing a wide range of concentrations to be tested.

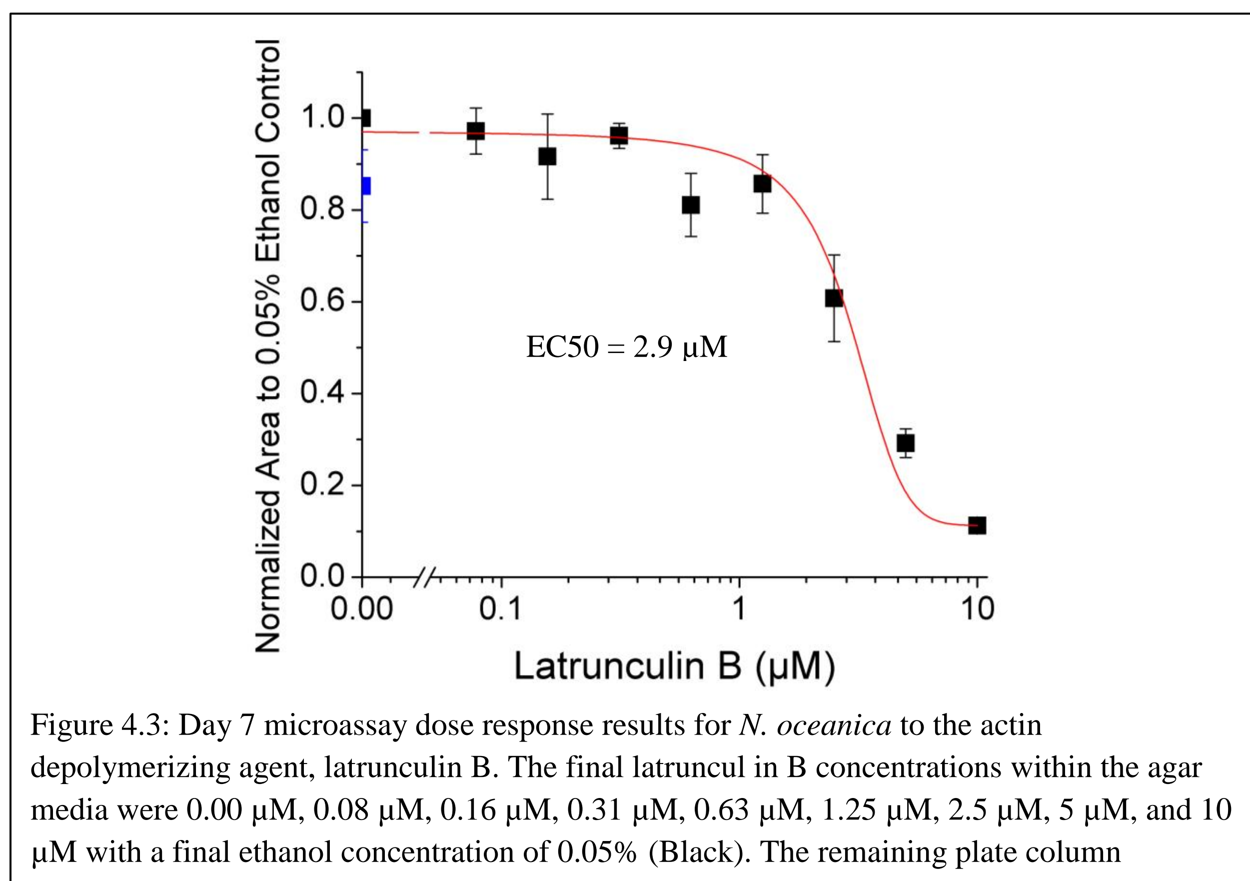

Figure 4.3: Day 7 microassay dose response results for *N. oceanica* to the actin depolymerizing agent, latrunculin B. The final latruncul in B concentrations within the agar media were 0.00 µM, 0.08 µM, 0.16 µM, 0.31 µM, 0.63 µM, 1.25 µM, 2.5 µM, 5 µM, and 10 µM with a final ethanol concentration of 0.05% (Black). The remaining plate column

4.4 Conclusion:

The 96 well microassay facilitates replicate testing of many conditions using small quantities of materials. If paired with automation, this assay could become a powerful tool for testing many media constituents quickly and efficiently. When compared to the 10mL liquid

culture, the microassay used 25X less material and displayed direct quantifiable growth in response to varying levels of test compounds. In terms of media optimization for liquid media, this microassay was limited; although correlations were similar between liquid and solid media for nitrate, they did not correlate for phosphate. On the other hand, one of the benefits of this method is its potential use as a rapid toxicity assay that overcomes many of the limitations of a recently reported similar method (Kviderova, 2010). Indeed, the current method showed that there was a definitive dose response of *N. oceanica* to latrunculin B. Therefore, considerably less drug or compound could be used to economically establish toxicity responses. Due to its simplicity and potential for automation, this assay may prove useful for multifactorial analysis that combines dose response to drug combinations, nutrients, and genetic modifications.

Chapter 5: An Algae Kinesin Phylogenetic Study

5.1 Introduction

Kinesins are motor proteins that travel along microtubules to transport vesicles, proteins, and chromosomes (Vale, 2003). The kinesin motor or head domain that interacts with microtubules is the conserved region of these molecules that defines the superfamily. These proteins are heavily involved in cell division and phragmoplast assembly for plants (Zhu & Dixit, 2011). In terms of understanding the growth and proliferation of oil producing algae, kinesins are important molecules to investigate because they play essential roles in cellular proliferation and cytoplasmic organization. If cell division can be manipulated to increase cell proliferation rates within an algae culture, or if conditions can be identified to produce dense cultures, lipids could be harvested quicker and at higher densities. Therefore a phylogenetic analysis of the kinesin super-family is important because it helps determine how many kinesins are present for a specific species as well as hypothesize the function of the kinesins present. Thus, this analysis provides insight to which kinesins might be important to manipulate to alter cellular proliferation.

Currently, there have been few sequenced algae genomes that are important to biodiesel production. Additionally, many of the protein models determined by the sequencing projects are still being developed and in many cases annotations are unreliable. However, the conserved nature of the kinesin motor head allows for reliable gene models of these regions and provides an initial estimate of how many kinesins are present within a genome. Together, this information allows for classes to be assigned within each of the kinesin families.

Therefore, as an addendum to our published manuscript annotating and describing the kinesin motor domains of *Physcomitrella patens*, the following algal species were included into

our phylogenetic analysis: *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea* C-169, *Chlorella variabilis* NC64A, and *Nannochloropsis gaditana* CCMP526 (Jinkerson et al., 2012, Shen et al., 2012, Merchant et al., 2007, JGI, 2009, Blanc et al., 2010). Using BLAST and searching the NCBI website, 37 kinesins were found for *C. reinhardtii*, 23 kinesins were found in *C. subellipsoidea* C169, 26 kinesins were found in *C. variabilis* NC64A, and 2 kinesins protein sequences were found for *N. gaditana*. These kinesins were added to the pool of *P. patens*, *A. thaliana*, *Homo sapiens*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and other key kinesins previously collected in order organize and annotate the algal kinesins.

5.2 Materials and Methods

Sequences were identified through BLAST with the *Arabidopsis* Kinesin 1 head against the *C. reinhardtii* version 4.3 genome sequence available from phytozome, the *C. subellipsoidea* C169 version 2.0 genome sequence and the *C. variabilis* NC64A version 1.0 genome sequence both available from JGI, and by searching for sequenced kinesin proteins available for *N. gaditana* CCMP526 (Jinkerson et al., 2012, Shen et al., 2012, Merchant et al., 2007, JGI, 2009, Blanc et al., 2010). Sequences were then added to the pool of *P. patens*, *A. thaliana*, *Homo sapien*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* kinesin head that were previously aligned and organized (Shen et al., 2012). Additionally, other kinesins important for kinesin nomenclature and identification were used to help order the tree more effectively (Lawrence et al., 2004). The species used consisted of *Xenopus laevis*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Mus musculus*, *Leishmania chagasi*, *Strongylocentrotus purpuratus*, *Emericella nidulans*, *Gallus gallus*, *Oryza sativa*, and *Cylindrotheca fusiformis*.

Alignments and trees were constructed the same as described in Shen et al. 2012. The pool of proteins were imported into Vector NTI Advance 11.5.1 (Invitrogen), and alignments were assembled with the included align X program. The default ClustalW parameters were used and alignments were imported to Geneious (Biomatters Ltd.).

Maximum likelyhood trees were assembled using the PhyML plugin in Geneious (Guindon *et al.*, 2010). Default parameters were used and subsequent trees were altered to only display nodes with more than 50% boostrap support. Nomenclature was continued as previously determined, and algae proteins were named with kinesin number followed by class signified by Roman numerals.

5.3 Results and Discussion

As displayed in Table 5.1, 37 kinesins were found for *C. reinhardtii*, 23 for *C. subellipsoidea* C169, 26 for *C. variabilis* NC64A, and only 2 could be found for *N. gaditana* CCMP526. The importance of these results shows the power of algae kinesins as 37, 23, and 26 proteins will be more manageable for functional studies than the 76 or 60 kinesins present in *Physcomitrella* or *Arabidopsis*, respectively (Table 5.1). Additionally, in comparison to an older phylogenetic study where version 2 of the *C. reinhardtii* genome was used, this analysis used the newer 4.3 version of the genome, and it displayed 14 more kinesins than previously reported (Table 5.1) (Richardson et al., 2006). Also, due to the lack of annotations for the *N. gaditana* genome, I expect that more than the two accessible kinesins are present within this genome, as a full list of predicted protein sequences have yet to be published.

In this study, Kinesins 3s and 10s will not be discussed because there are none of these proteins present within the algae genomes. Additionally, Kinesin 11 and the orphan kinesins will

be discussed together due to the ambiguity between identifying each class of the proteins. Each section will be separated by functional relevance of each class of kinesin.

Table 5.1: Number of algal kinesins ordered into family and class for each species with plant function included. Cr – Chlamydomonas reinhardtii, Cv - Chlorella variabilis NC64A, Cs - Coccomyxa subellipsoidea C169, Ng - Nannochloropsis gaditana CCMP526, Pp - Physcomitrella patens, At - Arabadopsis. Thaliana.

Kinesin Family	Functions	Cr	Cv	Cs	Ng	Pp	At
ARK		**1**	**3**	**1**	-	**5**	**3**
Class I	Polarized Growth	-	-	-		4	3
Class II	Unknown	1	3	1		1	-
Kinesin 1	Vesicle Trafficking	**1**	**1**	**1**	-	-	**1**
Kinesin 2	Flagella	**2**	**3**	**1**	-	**1**	-
Kinesin 4	Cellulose Deposition	**3**	-	**1**	-	**8**	**3**
Class I		1	-	1		5	3
Class II		1	-	-		3	-
Kinesin 5	Cell Division	**1**	**2**	**1**	-	**4**	**4**
Kinesin 6	Antiparallel MT Linkage	**1**	**1**	**1**	-	-	-
Kinesin 7		**3**	**2**	**1**	-	**7**	**14**
Class I	Organelle Transport	1	1	-		2	5
Class II	Cytokinesis	1	-	-		3	7
Class III	Kinetochore Capture	-	-	-		1	1
Class IV	Kinetochore Capture	-	-	-		1	1
Class V	Kinetochore Capture	1	1	1		-	-
Kinesin 8	Unknown	**1**	-	-	-	**3**	**2**
Class I		1				2	1
Class II		-				1	1
Kinesin 9	Flagella?	**3**	**1**	**2**	**1**	**3**	-
Class I		2	-	-	-	3	
Class II		1	1	1	-	-	
Kinesin 10		-	-	-		-	**2**
Kinesin 12		**1**	**2**	**3**	-	**18**	**6**
Class I	Phragmoplast	-	-	-		15	3
Class II	Phragmoplast	-	-	-		3	3
Class III	Unknown	-	1	1		-	-
Kinesin 13	Golgi Location	**1**	**1**	**1**	**1**	**3**	**2**
Kinesin14		**5**	**5**	**5**	-	**15**	**21**
Class I	Cell Division	2	2	2		2	4
Class II	Cytoskeleton Linkage	1	1	1		4	9
Class III		-	-	-		2	3
Class IV		-	-	-		1	2
Class V		-	-	-		2	2
Class VI	Cytoskeleton Organization	1	1	1		4	1

Table 5.1: Continued number of algal kinesins ordered into family and class for each species with plant function included. Cr – Chlamydomonas reinhardtii, Cv - Chlorella variabilis NC64A, Cs - Coccomyxa subellipsoidea C169, Ng - Nannochloropsis gaditana CCMP526, Pp - Physcomitrella patens, At - Arabadopsis. Thaliana.

Kinesin Family	Functions	Cr	Cv	Cs	Ng	Pp	At
Orphan	Regulatory Proteins	**14**	**5**	**5**	-	**9**	**2**
Class I	or	-	-	-		2	-
Class II	Potential pseudogenes	-	-	-		2	1
Class III		2	1	1		1	-
Class IV		2	-	-		4	1
Class V		2	1	1		-	-
Total		**37**	**26**	**23**	**2**	**76**	**60**

Cell Division

Kinesin 5

Kinesin 5s are the largest monophyletic group and have been found in all eukaryotic kingdoms (Richardson et al., 2006). A single Kinesin 5 can be seen for *C. reinhardtii* and for *C. subellipsoidea* C169, while two are present for *C. variabilis* NC64A (Table 5.1 and Figure 5.1). Previous research from mutations in yeast has shown that Kinesin 5 is heavily involved in mitosis and spindle formation (Ferenz *et al.*, 2010). Additionally, KSP in mammalian cells has been studied in oncology as a drug target due to its heavy involvement in mitotic spindle formation (Tao *et al.*, 2005). The conserved function of Kinesin 5 has also been seen in plants as it was determined in *A. thaliana* that Kinesin 5 was very important for spindle formation during mitosis (Bannigan *et al.*, 2007). As a result, it should be anticipated that the algae kinesins display similar functions and *C. reinhardtii* could easily be used to perform a molecular knockdown study to determine if the function continues to be conserved. Additionally, Kinesin 5 should be considered an area of interest when investigating cell division and turnover rate for algae.

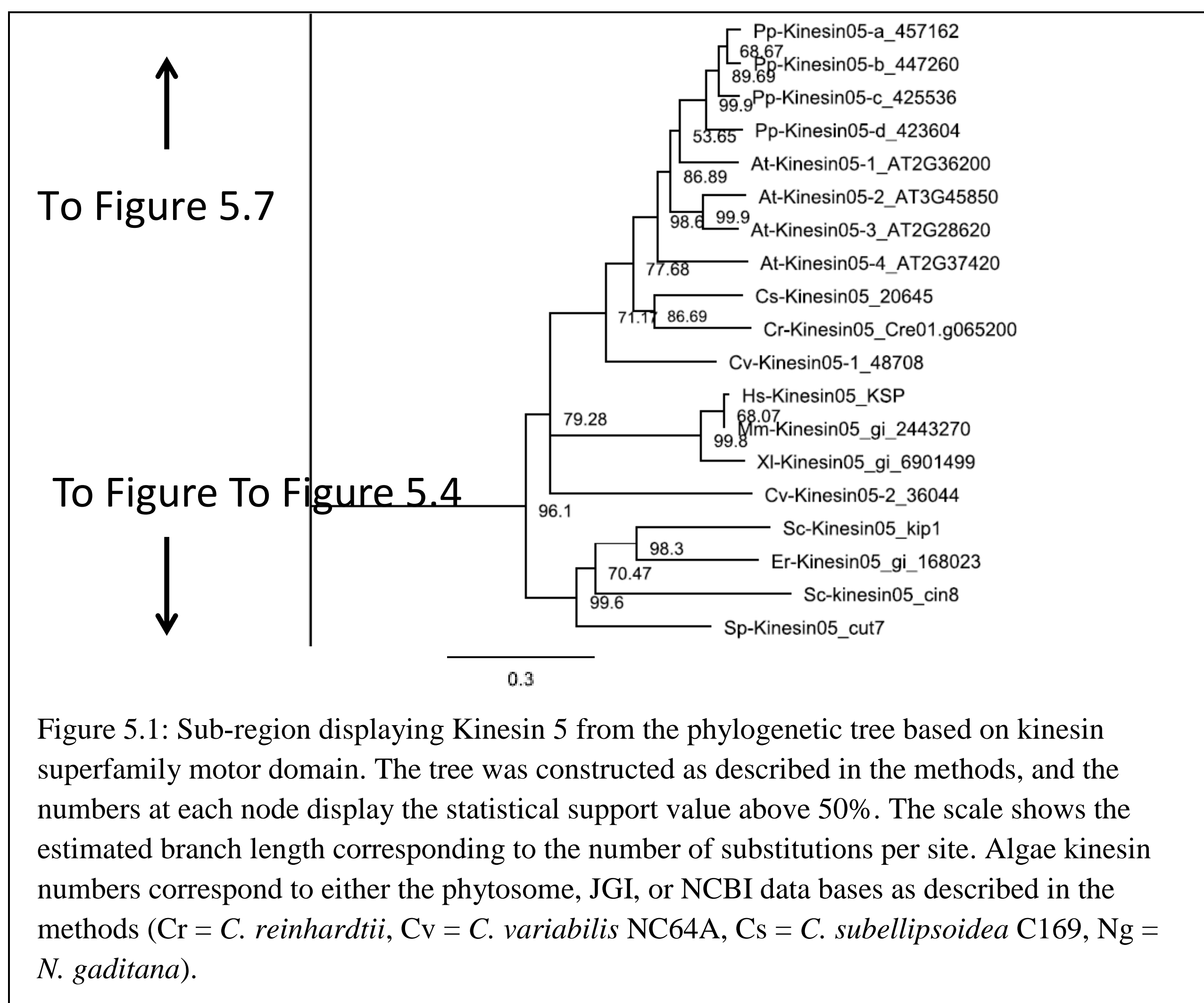

Figure 5.1: Sub-region displaying Kinesin 5 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

Kinesin 7 Class II and Class V

Kinesin 7 is an expanded group of kinesins in higher plants with 7 proteins in *Physcomitrella* and 14 in *Arabidopsis*. In the algae, only *C. reinhardtii* has a relatively expanded group as it contains 3 Kinesin 7's, while *C. subellipsoidea* C169 has 1, and *C. variabilis* NC64A has 2 Kinesin 7's (Table 5.1 and Figure 5.2). Class II of Kinesin 7 has been previously shown to important in cell division as previous work with the NACK kinesins from *A. thaliana* has shown that these proteins are very involved in cytokinesis (Tanaka *et al.*, 2004, Takahashi *et al.*, 2010, Sasabe *et al.*, 2011). The only classes II kinesin present form the algae is the *C. reinhardtii*

protein (Figure 5.2). Therefore, with only one protein present in the *C. reinhardtii*, genetic studies would greatly benefit Kinesin 7 class II function analysis (Figure 5.2).

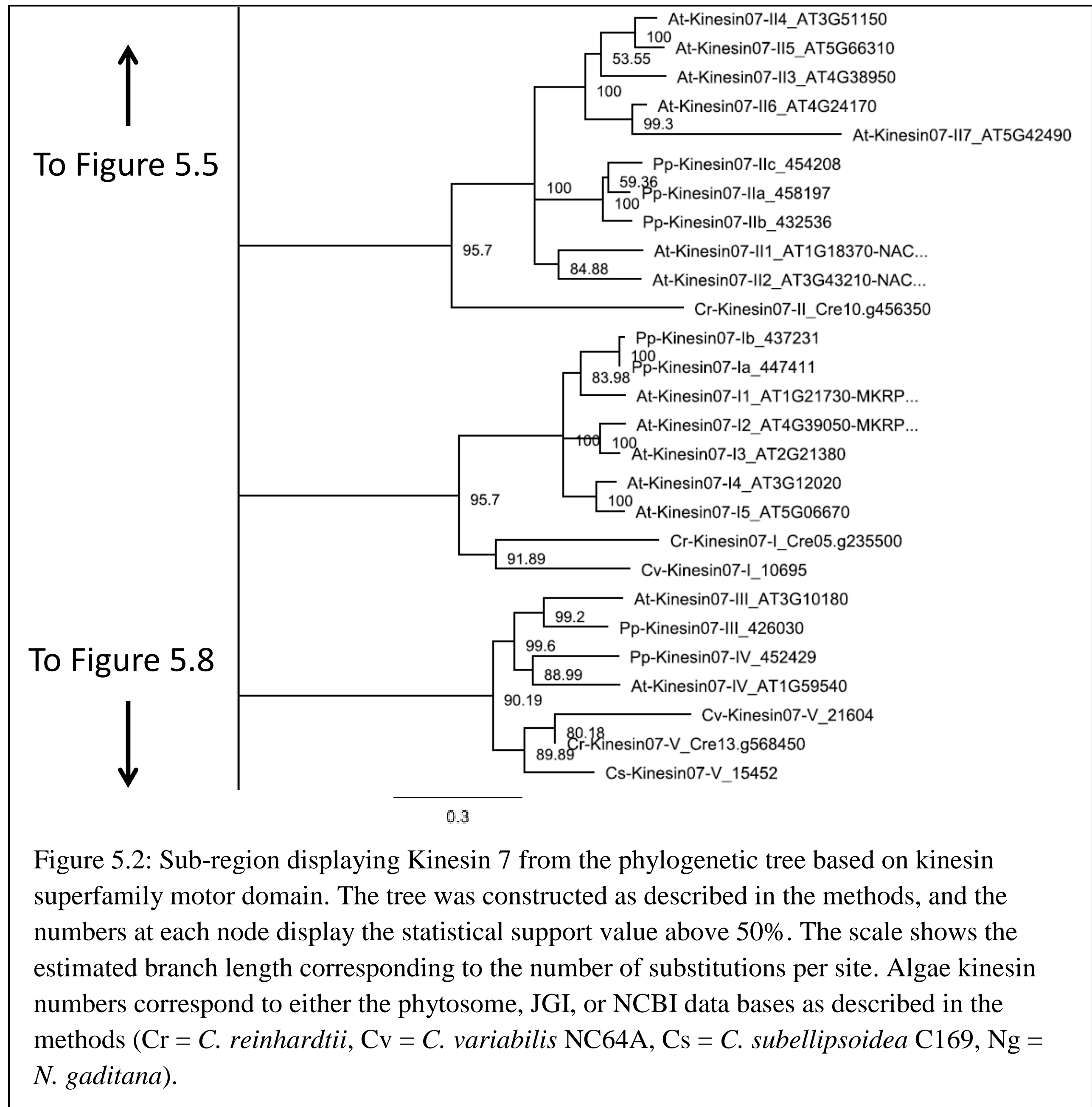

Figure 5.2: Sub-region displaying Kinesin 7 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

The Kinesin 7 Class V subfamily of algae kinesins is highly related to the class III and IV proteins present within *P. patens* and *A. thaliana* (Figure 5.2). Classes III and IV were initially separated due to their differences in the C-terminal domains, in which class III contains many

more and longer coiled coil domains (Shen et al., 2012). As a result, the algae proteins have been labeled class V due to the lack of information allowing the annotation of C-terminal domains. However, these classes are expected to be related to the CENPE kinesin (Shen et al., 2012), which is heavily involved in kinetochore capture (Weaver *et al.*, 2003). Therefore, these algae class II and class V proteins should be further studied and examined to determine their role in cell division.

Kinesin 14 Class I

Like Kinesin 7, Kinesin 14 is largely expanded in plants in comparison to other eukaryotic organisms. There are 5 Kinesin 14's each for *C. reinhardtii*, *C. subellipsoidea* C169, and *C. variabilis* NC64A, and every algae species have two class Is, one class II, one class VI, and 1 non class kinesin 14 (Table 5.1 and Figure 5.3). Although, the overall number of kinesin 14 in higher plants are much larger with 21 in *A. thaliana* and 15 in *P. patens*, this is the largest group of kinesins in each of these three algae species (Shen et al., 2012).

The function of class I has been shown to be involved in a variety of tasks across species. In mammalian cells, KIFC1 has displayed involvement at the nuclear membrane, in the biogenesis of the acrosome, and with vesicle transport (Nath *et al.*, 2007), while Kar3p has been shown to be important for nuclear fusion during sliding microtubule mediated *S. cerevisiae* mating (Yang & Sperry, 2003, Yang *et al.*, 2006, Meluh & Rose, 1990). In plants, ATL1 and ATK5 are important in *A. thaliana* for microtubule organization in mitosis and meiosis (Chen *et al.*, 2002, Ambrose *et al.*, 2005). Due to the assortment separating each species in this phylogenetic study of these kinesins (Figure 5.3), it is important for genetic studies to be performed on the algae kinesins in order to determine the function of the algae class I Kinesin

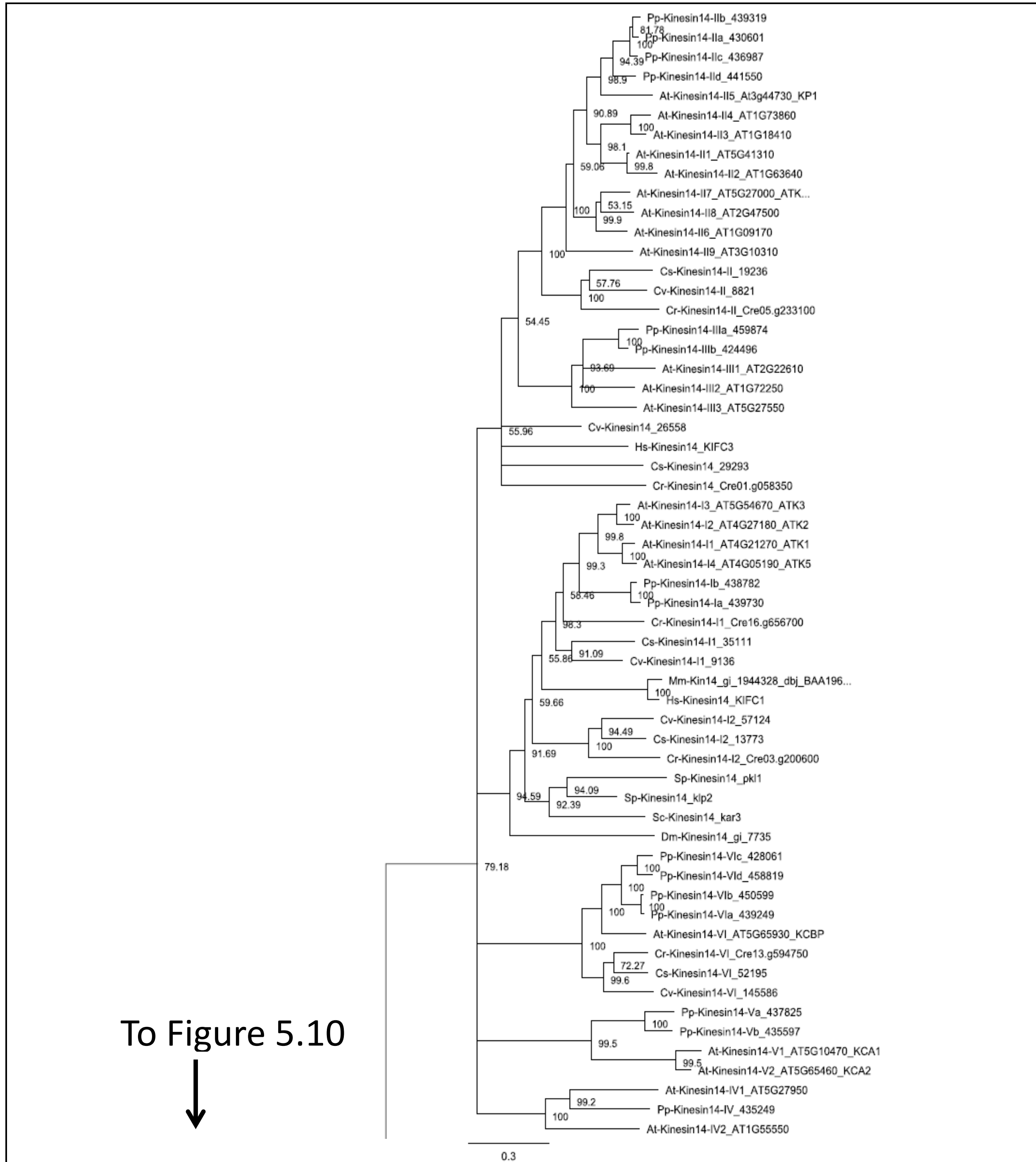

Figure 5.3: Sub-region displaying Kinesin 14 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

14's, but it should be anticipated that the function is more closely related to *A. thaliana*, organizing microtubules during mitosis and meiosis.

Flagella

Kinesin 2

Kinesin 2 motors are primarily involved in intraflagellar transport and have previously been studied in *C. reinhardtii* (Sloboda & Howard, 2007). As shown in Table 5.1 and Figure 5.4, *C. reinhardtii* has 2, *C. subellipsoidea* C169 has 1, and *C. variabilis* NC64A has 3 Kinesin 2's. It has been shown that the two motors for *C. reinhardtii* are directly involved with transport and signal transduction within the two flagella at the head of the organism (Pan & Snell, 2002, Pan & Snell, 2003). Additionally, the absence of Kinesin 2 in flowering plants, yeast, and invertebrates is consistent with previous studies and is expected due to the lack of flagella in these organism (Richardson et al., 2006). Furthermore, the human Kinesin 2 within the clade, KIF3, is heavily related to organ location, cilia function, and flagella function as a KIF3 defect will develop Kartagener's syndrome, which is characterized by immotile flagella, nonfunctioning cilia, mucus build up, and occasionally irregular organ placement (Hirokawa, 2000). Thus, it is reasonable to hypothesize that these kinesins always flagella related. However, *C. subellipsoidea* C169 and *C. variabilis* NC64A are nonmotile cells, and it is unclear if *Coccomyxa* sp. and *Chlorella* sp. are able to produce flagellated haploid cells known as zoospores. Therefore, the function of Kinesin 2 in these cell types is unknown, while in all other organisms, it is flagella and cilia related.

Kinesin 9

The overall function of the Kinesin 9 clade is unknown, however, it is expected to be comprised of cilia and flagella related proteins, much like Kinesin 2 (Miki et al., 2005). As a result, these 3 *C. reinhardtii*, 2 *C. subellipsoidea* C169, 1 *C. variabilis* NC64A, and 1 *N.*

gaditana motors (Table 5.1 and Figure 5.5) should be expected to be flagella related proteins. Previous work has shown in *C. reinhardtii* that the Kinesin 9 protein, Klp1, does have a direct relationship with the flagella (Bernstein *et al.*, 1994). Additionally, like Kinesin 2, Kinesin 9 is absent in flowering plants, yeast, and invertebrates providing further reasoning to suspect this clade as flagella and cilia related (Richardson et al., 2006). Moreover, these proteins are related to the two flagella for *C. reinhardtii*, and should be expected to be involved in the flagella of zoospores in other algae that produce them. For the non-motile algae in this study that might not produce zoospores, the function will remain unknown. Further genetic studies with *C. reinhardtii* or *N. gaditana* will help provide more insight to the unknown nature of Kinesin 9 motors.

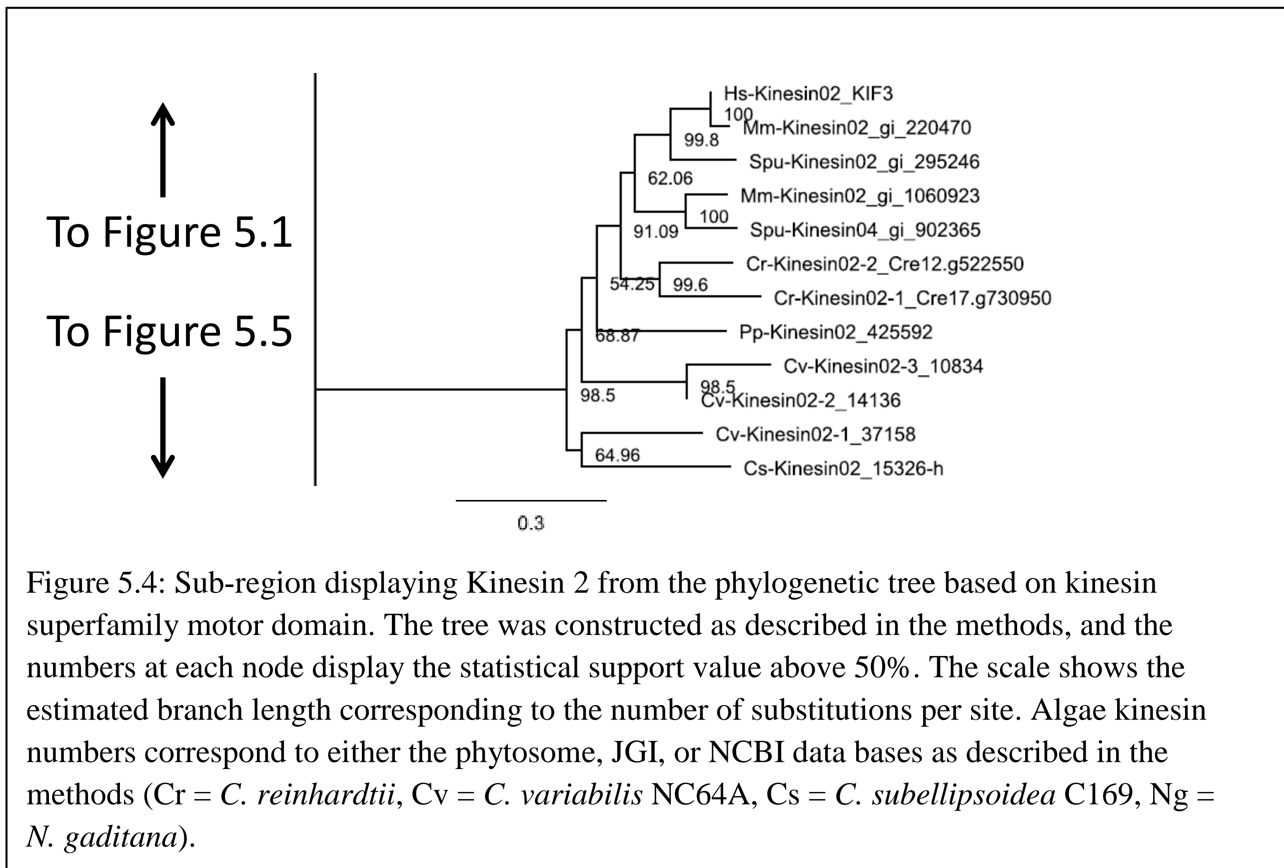

Figure 5.4: Sub-region displaying Kinesin 2 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

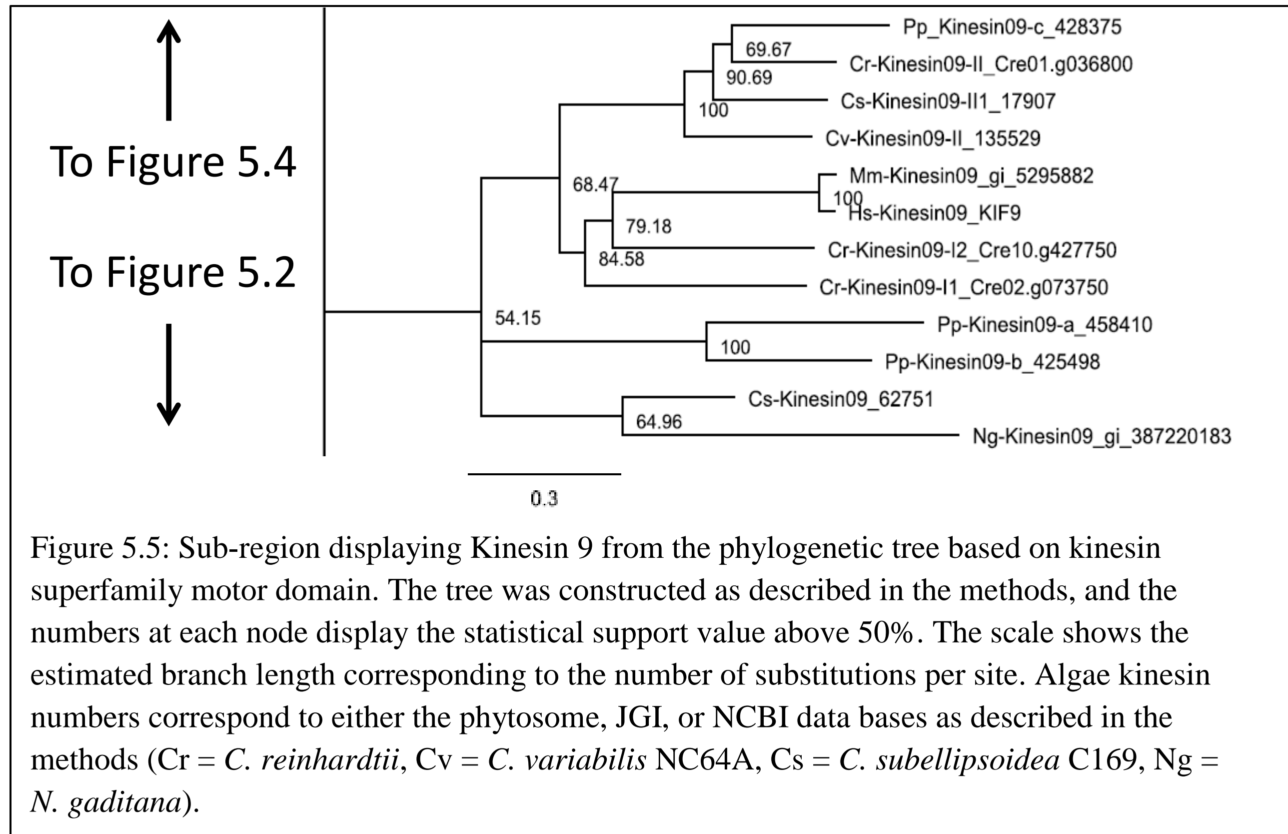

Figure 5.5: Sub-region displaying Kinesin 9 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

Organelle Transport and Plant Organization

Kinesin 1

Previous kinesin phylogenetic studies have shown distinct subfamilies of Kinesin 1 separating animal, fungal, plant, and atypical kinesins (Miki et al., 2005). This is consistent with my findings as the algae proteins appear to have a higher homology with the *A. thaliana* Kinesin 1, rather than the human and fungal Kinesin 1 proteins, as they are also separated by kingdom (Figure 5.6). There is a single Kinesin 1 each for *C. reinhardtii*, *C. subellipsoidea* C169, and *C. variabilis* NC64A (Table 5.1 and Figure 5.6). Research has shown that in neurons, the function of the Kinesin 1 motors are vesicle transport (Su *et al.*, 2004). Additionally, if there is a mutation with the human Kinesin 1 KIF5 proteins, people will develop spastic paraplegia, which is a disease that occurs due to inability to transport vesicles through long neurons leading to motor neuron degeneration (Ebbing *et al.*, 2008). As a result, it can be hypothesized that Kinesin 1 in

algae also is also involved with vesicle trafficking. However, with a lack of research on kinesin 1 in plants, the precise function has to still be determined.

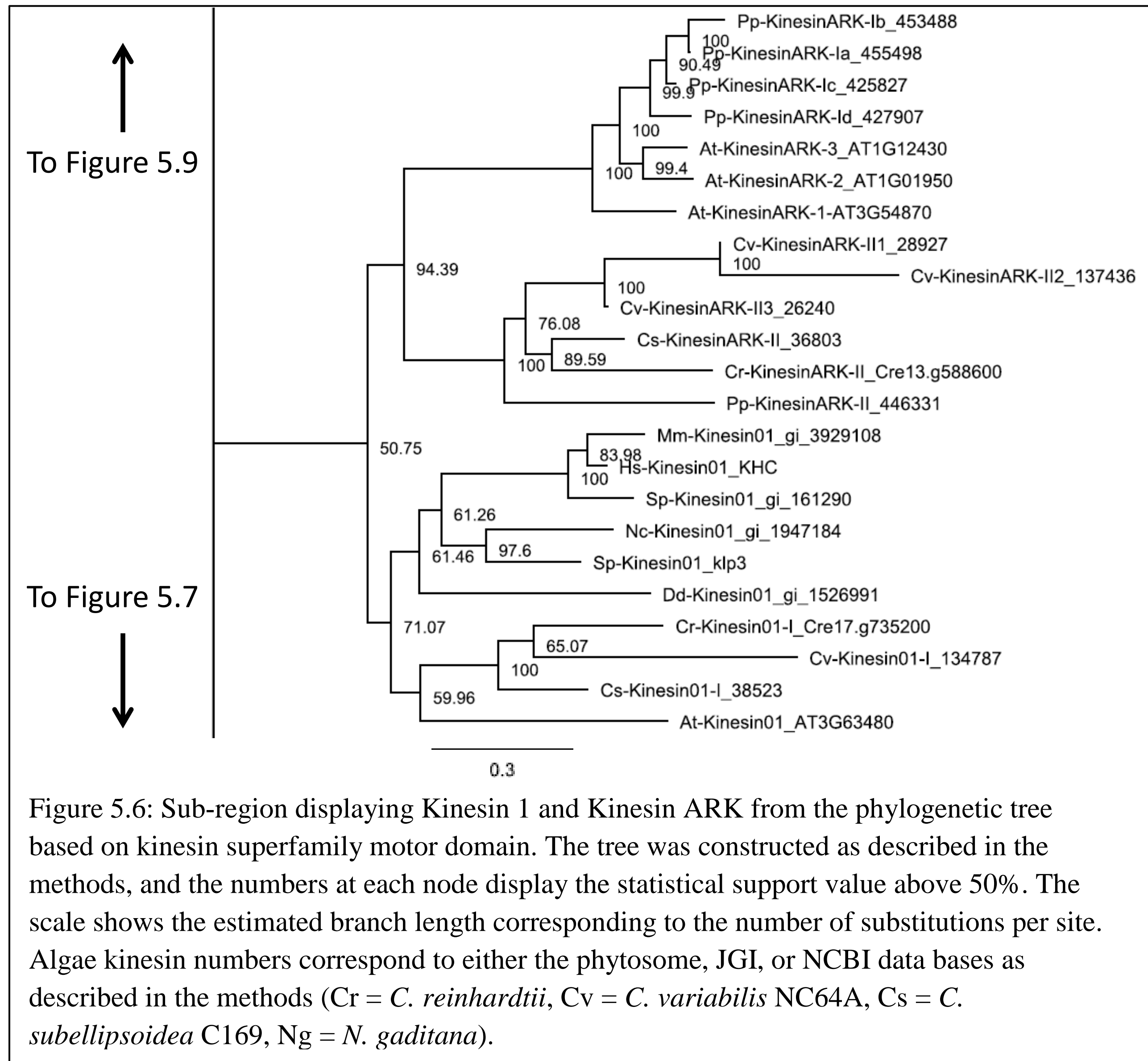

Figure 5.6: Sub-region displaying Kinesin 1 and Kinesin ARK from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

Kinesin 4

As displayed in the original paper, there are two distinct classes of Kinesin 4's. Within this clade, *C. reinhardtii* has 3 motors and *C. subellipsoidea* C169 has 4 motors (Table 5.1 and Figure 5.7), and *C. reinhardtii*, *C. subellipsoidea* C169, *H. sapien*, *P. patens* and *A. thaliana* were all present in class I, while only *P. patens* and *C. reinhardtii* were present in class II (Figure

5.7). The human Kinesin 4 has previously been shown to be heavily involved in midzone microtubule assembly during mitosis, while in *A. thaliana* and rice, Kinesin 4 proteins are used to help orient cellulose microfibrils (Kurasawa *et al.*, 2004, Zhang *et al.*, 2010, Zhong *et al.*, 2002). Therefore, it should be anticipated that these algal class I Kinesin 4's also help orient the cellulose microfibrils of the cell wall, since algae are closely related to the plants kinesins (Figure 5.7). However, the wide range of function between the animal kinesin and the plant kinesin proteins leave an uncertainty of the function for class II and the additional Kinesin 4 found in *C. reinhardtii* (Figure 5.7). Knockout studies with *C. reinhardtii* and *P. patens* would

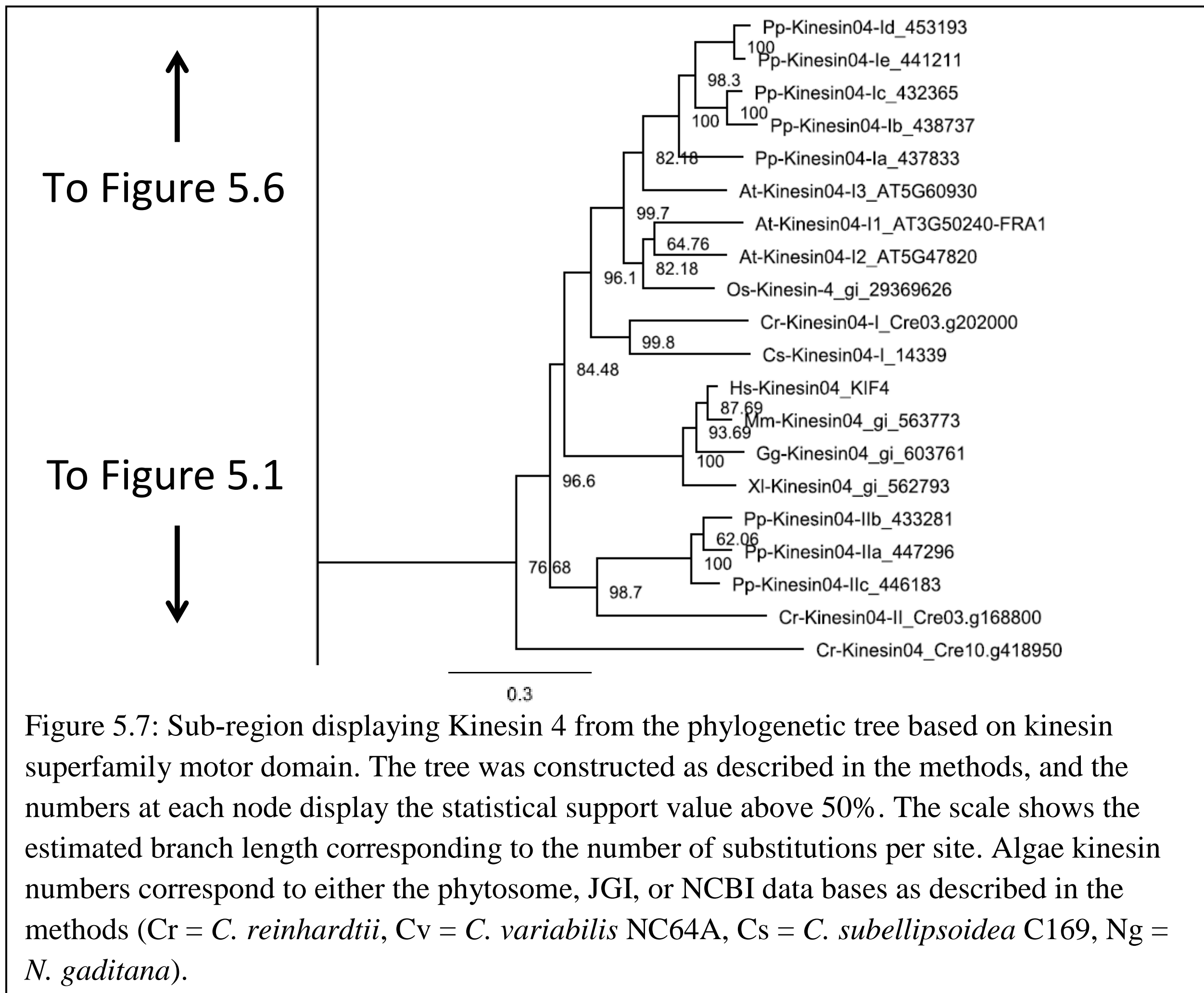

Figure 5.7: Sub-region displaying Kinesin 4 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

provide a great deal of information for the functions of Kinesin 4 in plants and potentially address the lack of class II and additional kinesin classes within humans and higher plants.

Kinesin 6

There is an example of Kinesin 6 for *C. reinhardtii*, *C. subellipsoidea* C169, and *C. variabilis* NC64A, which are all related to the human Kinesin 6, KIF23 (Table 5.1 and Figure 5.8). Pervious research of this human kinesin shows that Kinesin 6 is responsible for antiparallel linkage between microtubules and help position mitotic spindles (Nislow *et al.*, 1992). Therefore it is reasonable to predict that these algae Kinesin 6's will have similar function to the human protein. However, considering that there are no Kinesin 6's present within higher plants as determined by our study and previous research, a genetic knockout or knockdown of one of these algal proteins will be very beneficial in order to determine if plant Kinesin 6s have similar or different functions (Figure 5.8)(Miki et al., 2005).

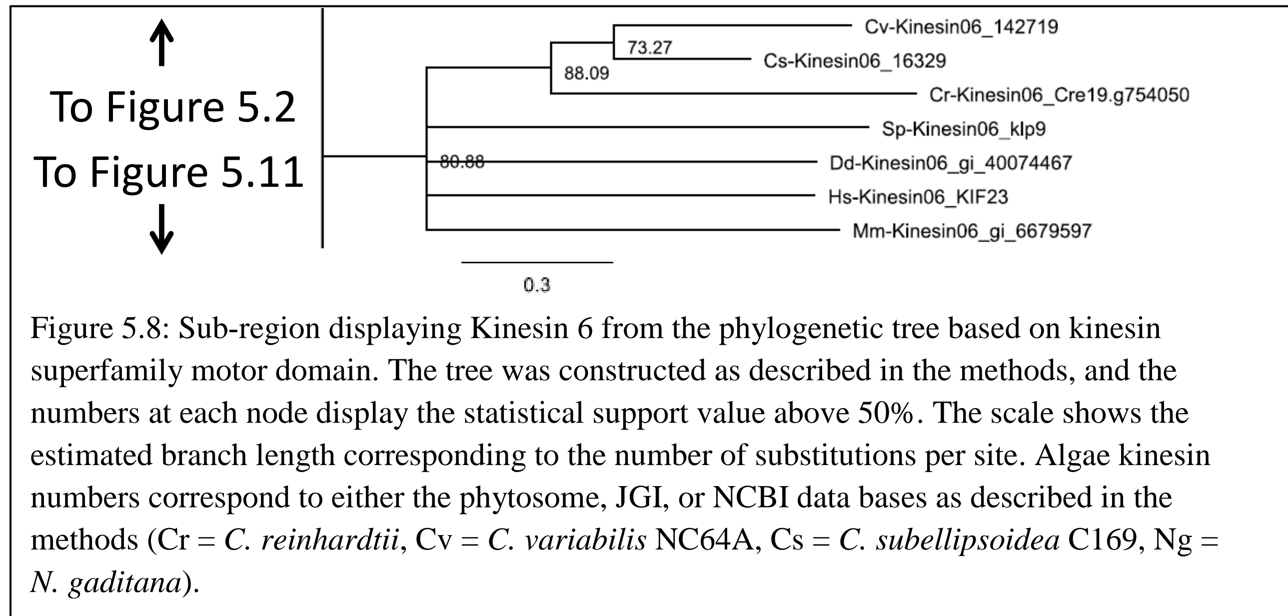

Figure 5.8: Sub-region displaying Kinesin 6 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

Kinesin 7 Class I

The remaining Kinesin 7's from the algae species are ordered into class I. These proteins from *C. reinhardtii* and *C. variabilis* NC64A are segregated from the *P. patens* and *A. thaliana* proteins (Figure 5.2), and previous research has shown that these class I proteins are involved with cytoplasmic organelle movement (Itoh *et al.*, 2001). Therefore, it is practical to expect class I algae function to be involved with organelle movement, but due to their distance, the function could differ and genetic studies need to be performed to specifically determine the function (Figure 5.2).

Kinesin 13

Kinesin 13 is very similar to Kinesin 8 in structure and function in other species as they are used for microtubule destabilization and organelle transport (Figure 5.9) as shown in animals and insects respectively (Homma *et al.*, 2003, Noda *et al.*, 1995). However, in *A. thaliana*, Kinesin 13 has a lysine rich neck sequence, which allows plants to display the function of golgi stack transportation (Lu *et al.*, 2005). Therefore, if the lysine rich neck sequence is present for the algae, it should be expected that the Kinesin 13's from *C. reinhardtii*, *C. subellipsoidea* C169, and *C. variabilis* NC64A have similar function to the *A. thaliana* kinesin. Although, due to the distance of the *N. gaditana* kinesin from the *A. thaliana* kinesin 13s (Figure 5.9), the protein may not possess this lysine rich neck sequence, and the function may not perform Golgi stack transport.

Kinesin 14 Class II and Class VI

The remaining class II, class VI, and non-class Kinesin 14's from *C. reinhardtii*, *C. subellipsoidea* C169, and *C. variabilis* NC64A are expected to be involved with cytoskeleton organization. Like class I of Kinesin 14, class II also has a strong distinction between species, as

A. thaliana, *P. patens*, and the algae are mostly separated from one another (Figure 5.3). In *A. thaliana* and *P. patens*, class II is the largest of the Kinesin 14s, and previous research in *A. thaliana* has shown that they are involved in organizing the cortical microtubule array, regulate mitochondrial functions, and is hypothesized to interact with F-actin through its Calponin Homology (CH) domain as shown in cotton and rice (Yang *et al.*, 2011, Tamura *et al.*, 1999, Xu *et al.*, 2009, Frey *et al.*, 2009). Due to the presence of these CH domains in *P. patens*, it is expected that these kinesins will perform similar functions (Shen et al., 2012). However, more

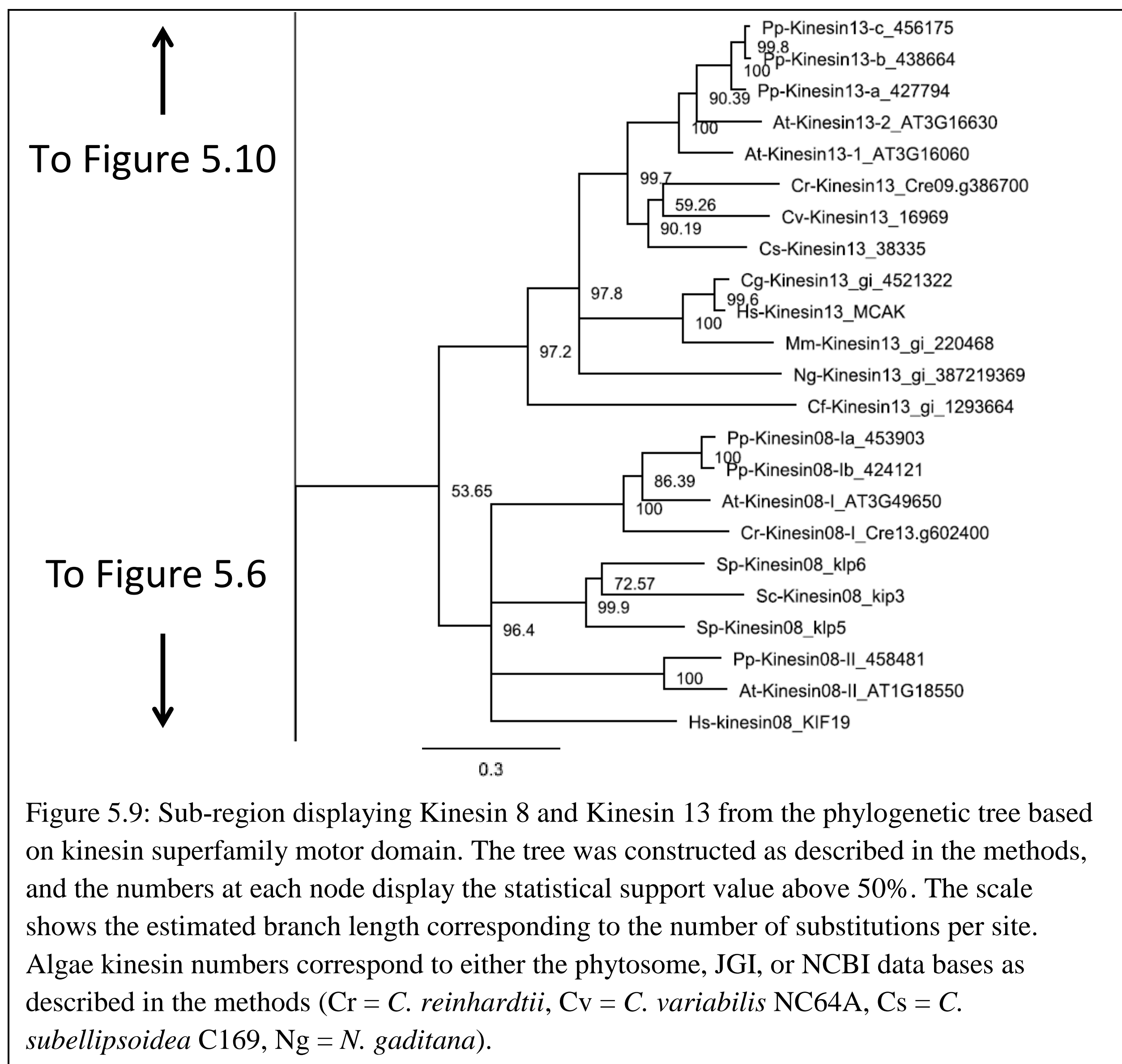

Figure 5.9: Sub-region displaying Kinesin 8 and Kinesin 13 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

research needs to be performed in order to create reliable gene models to determine the presence of these CH domains in the algae. Furthermore, if the CH domains are present, then it should be expected that these motors will display microtubule and microfilament linking activities.

As seen from Figure 5.3, the Kinesin 14 class VI proteins are segregated by species. The one *A. thaliana* protein present, KCBP, has been previously shown to contain a calmodulin binding domain, and it is involved in microtubule organization and bundling (Oppenheimer *et al.*, 1997, Bowser & Reddy, 1997, Vos *et al.*, 2000). Additionally, the *P. patens* proteins present contain a myosin tail homology domain, which could potentially allow it to act as a cross linker between microtubules and microfilaments (Shen et al., 2012). Although not much is known for the algae proteins, the *C. reinhardtii* Kinesin 14 class VI does contain the myosin tail homology domain (Richardson et al., 2006). Therefore, it should be expected that this domain will also be in the other algae species, since they are so closely related and the domain is present in all four *P. patens* proteins present. Furthermore, predictions for the functions of these class VI algae kinesins should be similar to the *P. patens* proteins and *A. thaliana* proteins, and these proteins should be involved in microtubule and microfilament crosslinking as well as microtubule bundling.

The final set of algae kinesins in this clade were not named with a class due to their lack of grouping with any other kinesins (Figure 5.3). The kinesin related the closest to these proteins is the human KIFC3 motor, which has been determined to be involved in Golgi transport and positioning (Xu *et al.*, 2002). Therefore, the closest prediction for these proteins would be Golgi interaction; however, overall function cannot be determined until genetic studies have been performed.

Unknown Plant Function

Armadillo Repeat Kinesins (ARK)

There was only 1 Armadillo Repeat Kinesin (ARK) within each of the genomes for *C. reinhardtii* and *C. subellipsoidea* C169, while *C. variabilis* NC64A contains 3 ARK kinesins (Table 5.1 and Figure 5.6). The ARK kinesin was initially characterized by its repeating 42 amino acid armadillo sequences within the C-terminus of the protein (Coates, 2003). However, all of these kinesins are related to the class II form of the motor head as determined by the class II *P. patens* ARK-LIKE protein (Figure 5.6). This is important because although this it has a high similarity between the motor heads of the ARK protein, the *P. patens* kinesin ARK-LIKE motor does not actually contain the armadillo repeats. Therefore, it is unlikely that the algae kinesins would contain the armadillo repeats also. Knockout studies have been performed in *A. thaliana*, which show that the function of ARK proteins with the armadillo repeats are important for polarized root hair growth (Sakai *et al.*, 2008, Yang *et al.*, 2007). As a result, these single celled spherical algal organisms have most likely never developed these repeats as there is no need to perform polarized growth, and it is probable to hypothesize that the ARKs found within the algae population are more related to the ancestral precursor form of the ARK protein and the armadillo repeat did not develop until much later on in its lineage. Genetic studies in *C. reinhardtii* and *P. patens* can help gain more insight to determine the role of the class II ARK proteins.

Kinesin 8

As stated in our previous phylogenetic study, nothing is known about Kinesin 8 in plants, but with one Kinesin 8 present in *C. reinhardtii*, genetic studies can be performed to help gain insight to the Kinesin 8 functions (Table 5.1 and Figure 5.9)(Shen et al., 2012). Previous research

has shown that Kinesin 8 is important for mitochondria transport in flies, microtubule destabilization in humans, and chromosome segregation during mitosis in yeast (Pereira *et al.*, 1997, Du *et al.*, 2010, West *et al.*, 2002). However, Kinesin 8 is separated into classes segregating the plant and animal proteins from the fungi proteins, making it difficult to determine likely Kinesin 8 functions for plants (Figure 5.9). Therefore, genetic studies need to be performed in order to assign specific functions to these kinesins.

Kinesin 12

The algae Kinesin 12's are interesting due to their lack of subfamily placement within the group (Figure 5.10). Previous research has shown that in animal cells, Kinesin 12 is important for bipolar spindle assembly and neuron development (Tanenbaum *et al.*, 2009, Buster *et al.*, 2003). In higher plants, both classes of Kinesin 12 are important for phragmoplast assembly and orientation (Lee & Liu, 2000, Pan *et al.*, 2004, Muller *et al.*, 2006). However, the algae kinesins have formed a third class and the remaining proteins are non-class Kinesin 12's (Figure 5.10). Therefore, it is very difficult to determine the function of these kinesins as Kinesin 12 has scatter functions across kingdoms, and the algae proteins do not align with the higher plants (Figure 5.10). Further research needs to be conducted to declare a function for these algae kinesins.

Kinesin 11 and Orphans

All other algae proteins in this study have been labeled as orphan kinesins due to a lack of organization within the tree (Figure 5.11). As shown in our initial phylogenetic study, there were four orphan classes that were grouped together, and the algae was grouped into class III and IV, however, this new tree also created a new class V that encompasses of only algae proteins (Figure 5.11). All other remaining proteins were classified only as orphan. Also, it should be noted that it is possible that some of these orphans could be pseudogenes. For example, the *P.*

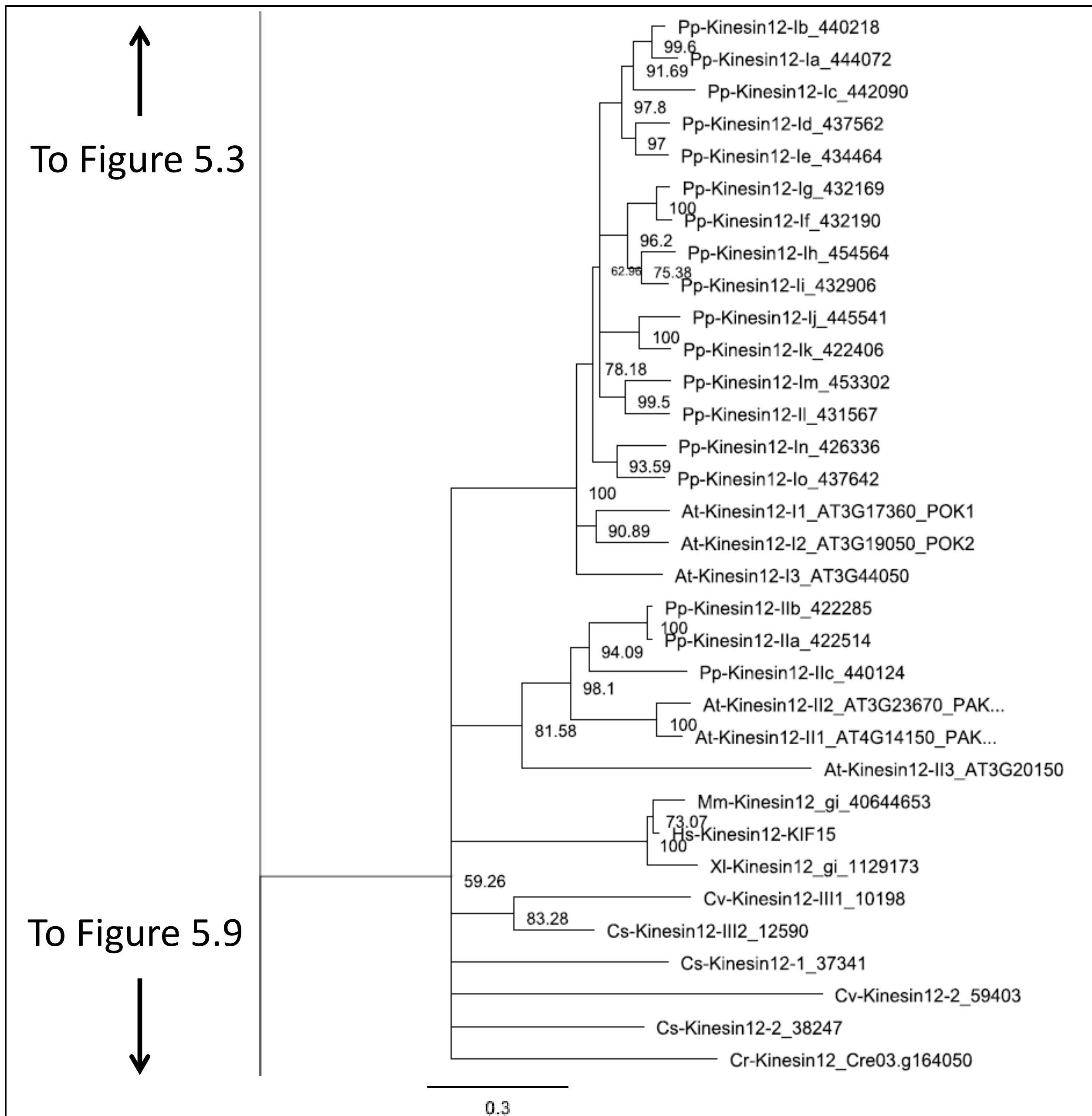

Figure 5.10: Sub-region displaying Kinesin 12 from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = *C. reinhardtii*, Cv = *C. variabilis* NC64A, Cs = *C. subellipsoidea* C169, Ng = *N. gaditana*).

patens class III protein that defines the group could potentially be a pseudogene as *P. patens* has a well annotated genome, yet this proteins has an incomplete gene model and cannot be assigned to one of the major families (Shen et al., 2012). It will be interesting to see if these algae genes that cluster to class III are functioning proteins or if they are also pseudogenes.

Class IV of the orphans is interesting because the motor domains are more related to our myosin out group than they are any of the kinesins in this study (Figure 5.11). Additionally, the motor heads contain coiled coil domains, which suggests that they are nonfunctional motors and function similar to the yeast kinesin 11, Smy1p which is suggested to regulate myosin V (Lillie & Brown, 1998, Beningo *et al.*, 2000). Therefore, these orphan kinesins or potential kinesin 11s display relatively low homology to one another (Figure 5.11), but their lack of functionality as a motor suggests that they could be a part of the same family regulating other proteins within the cell.

As for the rest of the orphans, including the newly named class V subfamily containing all algae kinesins, nothing is known (Figure 5.11). Their lack of grouping makes it difficult to decipher if they are pseudogenes, functional motors, or related to kinesin 11. Moreover, these proteins are problematic to assign a function and more research needs to be conducted.

5.4 Conclusion

Although much more research needs to be performed to gain further insight into algae kinesins, what I have presented here provides a good starting point for choosing specific kinesin proteins to study cellular processes. In terms of cell turnover rate and cell division, it is important to focus on algae Kinesin 5, Kinesin 7 class II and V, and Kinesin 14 class I. Outside of studying algal cell division, these algae kinesins can help determine the function of the ARK proteins without the ARK motif, how Kinesin 4 is used for cellulose deposition in plants, or help

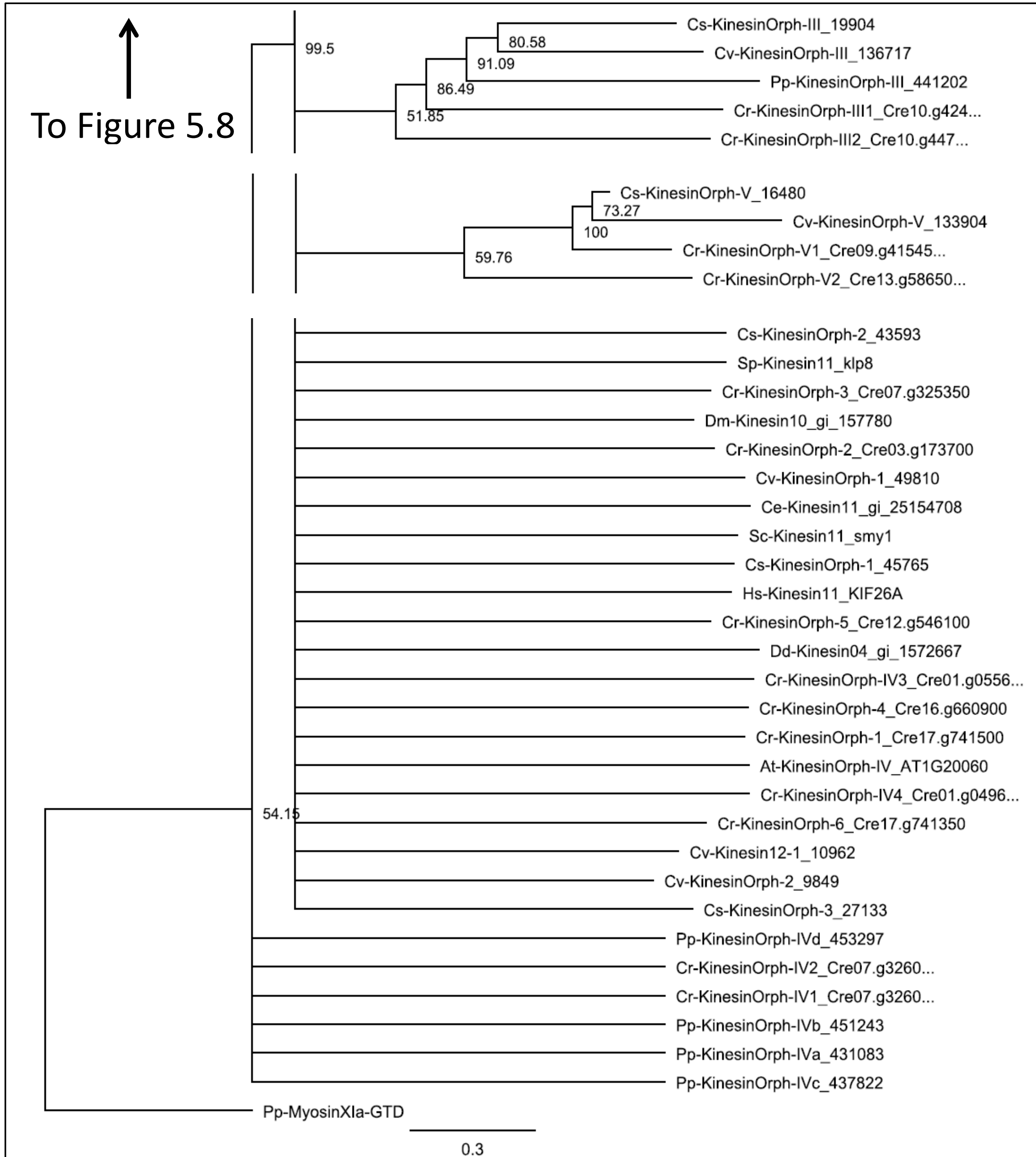

Figure 5.11: Sub-regions displaying potential kinesin 11 and orphan kinesins from the phylogenetic tree based on kinesin superfamily motor domain. The tree was constructed as described in the methods, and the numbers at each node display the statistical support value above 50%. The scale shows the estimated branch length corresponding to the number of substitutions per site. Algae kinesin numbers correspond to either the phytosome, JGI, or NCBI data bases as described in the methods (Cr = C. reinhardtii, Cv = C. variabilis NC64A, Cs = C. subellipsoidea C169, Ng = N. gaditana).

determine the function of the plant Kinesins 8 and 9, which are largely unknown. The algae kinesins can also provide awareness as to which orphan groups are pseudogenes, which are motor proteins, and which are nonfunctional regulatory proteins. Lastly, it will be interesting to study algae Kinesin 6 motors as they are expected not play a role in flagella or cilia function, yet they are missing in higher plants. Overall, assigning families to these algae motors allows for a better understanding of the kinesin superfamily, how these proteins can be studied further in the future, and identifies potential motor candidates for research in algal cell division.

Appendix

Table A: List of transformation protocols for various eukaryotic photosynthetic microorganisms and published reference.

Phylum	Type	*Genus species*	Transformation	Ref
Chlorophyta	Green	*Chlamydomonas reinhardtii*	Agitation	(Dunahay, 1993)
Chlorophyta	Green	*C. reinhardtii*	Agitation	(Kindle, 1990)
Chlorophyta	Green	*C. reinhardtii*	Bombardment	(Kindle et al., 1989)
Chlorophyta	Green	*C. reinhardtii*	Agrobacterium	(Kumar *et al.*, 2004)
Chlorophyta	Green	*C. reinhardtii*	Electroporation	(Brown et al., 1991)
Chlorophyta	Green	*C. reinhardtii*	Electroporation	(Shimogawara et al., 1998)
Chlorophyta	Green	*Chlorella ellipsoidea*	Electroporation	(Chen et al., 2001)
Chlorophyta	Green	*C. ellipsoidea*	Protoplast / PEG Mediated	(Jarvis & Brown, 1991)
Chlorophyta	Green	*Chlorella saccharophila*	Bombardment	(Dawson et al., 1997)
Chlorophyta	Green	*C. saccharophila*	Protoplast / Electroporation	(Maruyama et al., 1994)
Chlorophyta	Green	*Chlorella vulgaris*	Protoplast / PEG Mediated	(Hawkins & Nakamura, 1999)
Chlorophyta	Green	*C. vulgaris*	Electroporation	(Chow & Tung, 1999)
Chlorophyta	Green	*Chlorella kessleri*	Bombardment	(El-Sheekh, 1999)
Chlorophyta	Green	*Chlorella sp.*	Electroporation	(Wang et al., 2007)
Chlorophyta	Green	*Haematococcus pluvialis*	Bombardment	(Teng et al., 2002)
Chlorophyta	Green	*Volvox carteri*	Bombardment	(Jakobiak et al., 2004)
Chlorophyta	Green	*V. carteri*	Bombardment	(Schiedlmeier et al., 1994)
Chlorophyta	Green	*Ulva lactuca*	Protoplast / PEG Electroporation	(Huang et al., 1996)
Chlorophyta	Green	*Dunaliella viridis*	Electroporation	(Sun et al., 2006)
Chlorophyta	Green	*Dunaliella salina*	Electroporation	(Sun et al., 2005)
Chlorophyta	Green	*D. salina*	Bombardment	(Tan et al., 2005)
Heterokontophyta	Green	*Nannochloropsis oculata*	Protoplast / Electroporation	(Chen et al., 2008)
Heterokontophyta	Green	*Nannochloropsis* sp.	Electroporation	(Kilian et al., 2011)
Heterokontophyta	Diatom	*Thalassiosira pseudonana*	Bombardment	(Poulsen *et al.*, 2006)
Heterokontophyta	Diatom	*Thalassiosira weissflogii*	Bombardment	(Falciatore *et al.*, 1999)
Heterokontophyta	Diatom	*Phaeodactylum tricornutum*	Bombardment	(Apt *et al.*, 1996)
Heterokontophyta	Diatom	*P. tricornutum*	Bombardment	(Zaslavskaia *et al.*, 2000)
Heterokontophyta	Diatom	*P. tricornutum*	Bombardment	(Zaslavskaia *et al.*, 2001)

Table A: Continued list of transformation protocols for various eukaryotic photosynthetic microorganisms and published reference.

Phylum	**Type**	***Genus species***	**Transformation**	**Ref**
Heterokontophyta	Diatom	*Navicula saprophila*	Bombardment	(Dunahay *et al.*, 1995)
Heterokontophyta	Diatom	*Cyclotella cryptica*	Bombardment	(Dunahay et al., 1995)
Heterokontophyta	Diatom	*Cylindrotheca fusiformis*	Bombardment	(Fischer *et al.*, 1999)
Heterokontophyta	Diatom	*C. fusiformis*	Bombardment	(Poulsen & Kroger, 2005)
Rhodophyta	Red	*Cyanidioschyzon merolae*	Electroporation	(Minoda *et al.*, 2004)
Rhodophyta	Red	*Porphyra yezoensis*	Agrobacterium	(Cheney, 2001)
Rhodophyta	Red	*Porphyridium* sp.	Bombardment	(Lapidot et al., 2002)
Rhodophyta	Red	*Gracilaria changii*	Bombardment	(Gan *et al.*, 2003)
Dinoflagellate	Protist	*Amphidinium sp.*	Agrobacterium	(ten Lohuis & Miller, 1998)
Dinoflagellate	Protist	*Symbiodinium microadriaticum*	Agrobacterium	(ten Lohuis & Miller, 1998)
Euglenozoa	Green	*Euglena gracilis*	Bombardment	(Doetsch et al., 2001)

Table B: List of constructed and borrowed plasmids used for transformation experiments. Ubi – ubiquitin promoter, 35S – 35S mosaic virus promoter, βTP – Chlamydomonas beta tubulin promoter, VCP1 – Nannochloropsis violaxanthin/chlorophyll a-binding protein promoter, SuSy – Physcomitrella sucrose synthase with GFP, GFP – green fluorescent protein, mYFP – monomeric yellow fluorescent protein, Hygro – hygromycin B resistance, H8 – hygromycin B resistance

Plasmid	Resistance	Fluorescence	Acquisition
Ubi::SuSy	Hygromycin B	GFP	Yen Liu – Vidali Lab
Ubi::2X GFP	Hygromycin B	2X GFP	Constructed – GCS
Ubi::6X GFP	Hygromycin B	6X GFP	Constructed – GCS
Ubi::mYFP	Hygromycin B	mYFP	Constructed – GCS
35S::mYFP	Hygromycin B	mYFP	Constructed – GCS
2X 35S::mYFP	Hygromycin B	mYFP	Constructed – GCS
βTP::Hygro	Hygromycin B	No	Jason Brown – Umass Medical
VCP1::H8	Hygromycin B	No	Bert Vick – Aurora Algae

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