Identification and Localization of Carbon Concentrating Mechanism Components in *Chlamydomonas reinhardtii*

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Abstract

C_3 carbon fixing plants have limited photosynthetic capabilities. This is partially due to the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO, which wastes a lot of energy to photorespiration. Algae often experience fluctuations in inorganic carbon levels that alter the availability of materials to maintain sufficient RuBisCO function. To combat this problem, algae have evolved a Carbon Concentrating Mechanism (CCM), to elevate the internal concentration of CO_2 in the vicinity of RuBisCO. *Chlamydomonas reinhardtii,* a versatile model organism, both autotrophic and heterotrophic in nature, operates an efficient CCM. However, there is little known about the precise roles and functions of most candidate components of the CCM. Through the creation of a high throughput fluorescence-tagging pipeline in combination with protein localization technologies, this study helped to understand the functions and locations of specific CCM genes.

Table of Contents

Figures and Tables

Introduction:

As climate changes and population increases, the ability to maintain a stable food supply becomes increasingly difficult. With increasing temperatures and weather irregularity, C_3 plants, which encompass most crop plants, do not have an effective way of preserving energy and materials without increasing photorespiration and limiting other life processes. The Carbon Concentrating Mechanism (CMM), found in a variety of microalgae, cyanobacteria, C_4, and CAM plants, maximizes the photosynthetic output in plants' limited conditions.

The CCM increases the internal concentration of CO_2 in the vicinity of ribulose-1-5 bisphosphate carboxylase/oxygenase (RuBisCO), an enzyme that catalyzes the carboxylation of RuPB. This is the primary step in the assimilation of CO_2 into the photosynthetic carbon cycle. Unfortunately, RuBisCO also has an affinity for oxygen. At atmospheric levels of CO_2, RuBisCO can function at about 25% of its enzymatic capacity (Moreney, 2007). In many cases, RuBisCO fixes O_2, instead of executing its intrinsic function in photosynthesis, fixing CO_2. The result is photorespiration, and the creation of 2-phosphoglycolate (2-PG), which is useless to the cell, and can inhibit chloroplast function. Because carbon, nitrogen, and energy loss are associated with photorespiration, scientists continue to try to identify mutants with reduced photorespiration and high photosynthetic yield (Peterhansel, 2010).

Pollock et al. in 2003 generated a mutant that lacked the gene for RuBisCO Activase (Rca) the enzyme that catalyzes the activation of RuBisCO *in vivo* by removing inhibitory sugar phosphates. Results showed that the mutant grew at only 60% of the previous photosynthetic capacity, and that the Carbon Concentrating Mechanism partially compensated for the absence of an active Rca in *Chlamydomonas reinhardtii*. This suggests that a functional CCM should compensate for the loss of Rca in a low-CO_2 atmosphere. This is one of many novel mutants that have been studied.

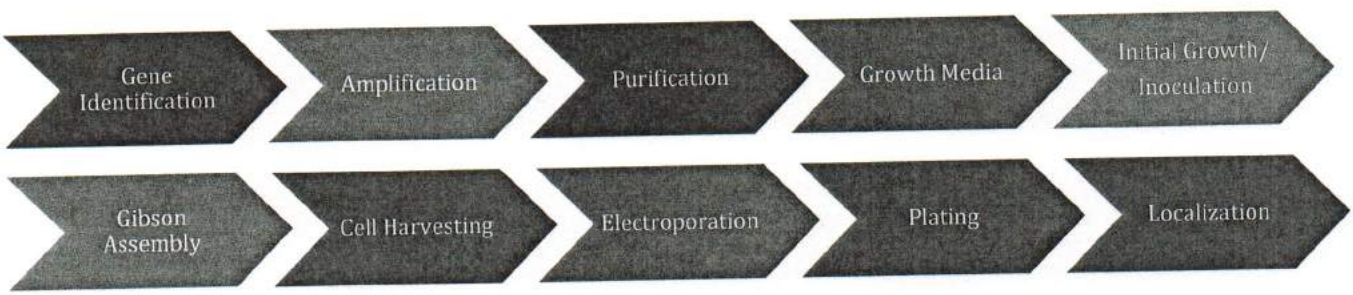
Benefits of having a CCM include an increased tolerance to low concentrations of inorganic carbon, reduced photorespiration, and a greater tolerance to water stress. McGrath et al. in 2014 created models to predict the benefits of engineering a full cyanobacteria CCM into a C_3 leaf. The simulations showed that increasing leaf

photosynthesis by 25% through artificial elevation of CO_2 in open-air conditions could increase the yield of soybeans by 15% and water use efficiency by 20%. Additionally, there would be a potential 60% increase in light saturated CO_2 uptake correlating to an additional 36% increase in yield. Although the molecular components of the CCM have been studied extensively in Cyanobacteria, there has been no significant advance in the understanding of the eukaryotic CCM.

Chlamydomonas reinhardtii, a eukaryotic green alga, has a very similar photosynthetic apparatus to that of land plants. It is unicellular, making high throughput experiments more feasible, and has a previously sequenced genome. Additionally, since Chlamydomonas can survive off of acetate in the absence of light, photosynthetic mutants can be generated without death of the organism.

C. reinhardtii has the best-characterized CCM, but there are still large gaps in our knowledge of the cellular components, in their location and how inorganic carbon flows through the cell. Through the creation of a high throughput fluorescence-tagging pipeline, in combination with protein localization technologies, this research aimed to fill in these gaps, and contribute to the creation of a viable CCM mechanism for C3 plants.

Methodology: High Throughput Tagging Pipeline (HTTP)



Gene Identification

Putative CCM genes were identified from a variety of sources, including multiple genome saturated mutant screens done by Leif Pallesen (Carnegie Institute), literature, and collaboration with STITT.

Amplification

These mutants were amplified using Polymerase Chain Reaction (PCR). Standard touchdown PCR was used at first for each gene. Initial annealing temperature was gradually reduced over subsequent cycles, decreasing from 72°C to 66°C. The advantage of using touchdown PCR is to increase efficiency and reduce unspecific products. The PCR master mix also contained 6% Dimethyl Sulfoxide (DMSO), and ran extension times that averaged 1000 base pairs per 30 seconds.

After PCR, products were run through the Gel Electrophoresis to ascertain that the PCR products were the correct sizes. Because of *C. reinhardtii's* guanine-cytosine (GC) rich nature, human error, and malfunctions in the creation of primers, many genes were unable to amplify correctly. To combat this, annealing temperatures, extension time, and DMSO concentrations were altered. The addition of Betaine was also tried. In many cases these alterations helped rescue previously failed primers.

Purification

Next, the MiniElute Gel Extract Kit was used to purify the genomic DNA of interest. The purified products were run against samples of known concentration to determine DNA concentration. (See Figure 1)

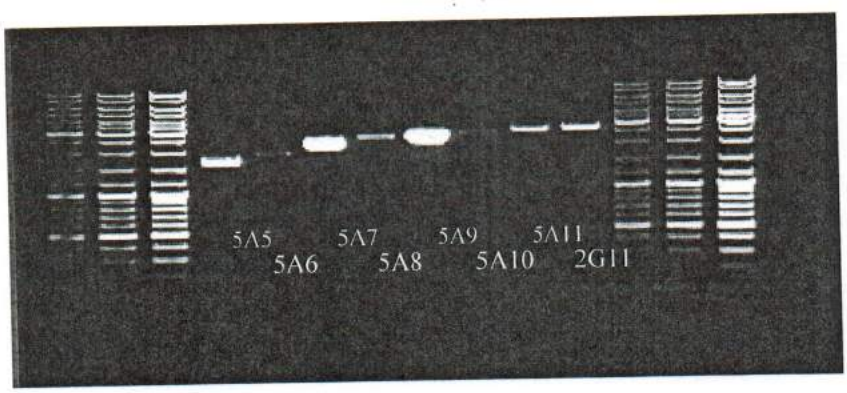


Figure 1: 8 purified primer products run to quantify DNA concentration.

Growth Media

Four liters of TAP media were created and autoclaved, with the stir bar in it and a normal cap for a liquid 120 cycle. Some was then mixed with agarose and ampicillin for selection, and then poured on plates. (See Table 1)

Initial Growth/ Inoculation

400 mL of culture in a 2L conical flask was inoculated from a fresh single colony. The cultures were then grown to a density between $5x10^6$ to $5x10^7$ cell/mL. Doubling time was estimated at about 8 hours. Cells were harvested and streaked on LB plates, then put in a 37C incubator over night.

4	L	medium	
40	ml	Tris acetate	
100	ml	TAP salts	
1.5	ml	phosphate solution	
4	ml	EDTA-Na2 @ 25 mM	
4	ml	(NH4)6Mo7O24 @ 28.5 μM*	
4	ml	Na2SeO3 @ 0.1 mM	
4	ml	Zn×EDTA @ 2.5 mM	
4	ml	Mn×EDTA @ 6 mM	
4	ml	Fe×EDTA @ 20 mM	
4	ml	Cu×EDTA @ 2 mM	

Table 1: Materials used to create 4 liters of TAP growth media

Gibson Assembly

A Gibson Assembly was used to clone *E.coli* to contain a number of necessary components needed for transformation. Plasmid sequences were checked with Eco-RV, and run on a gel to determine proper base pair size.

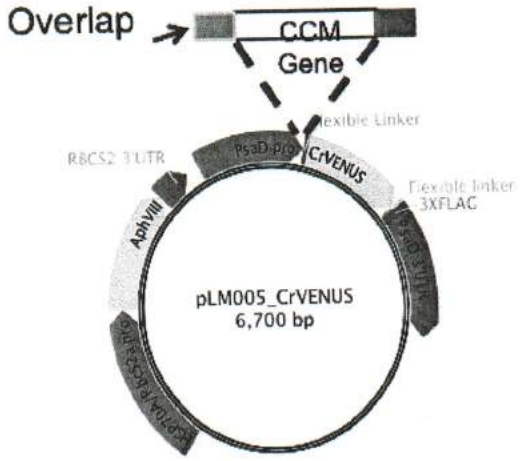


Image provided courtesy of Dr. Luke Mackinder

Figure 2: This is an example of a plasmid created by the Gibson Assembly. Genes of interest are cloned in frame with a fluorescent protein, venus. The plasmid was engineered to include a paromomycin resistance gene (AphVIII), for selection of successful *C.reinhardtii* transformants.

Harvesting

Cultures were aliquoted into 10 centrifuge bottles, and spun down for 4 minutes at 1000x g. Supernatants were discarded, and pellets were combined with 15mL of TAP Sucrose 40 mM. Cells were then diluted to $2x10^8$.

Electroporation

250uL of transformation mixture was dispensed into 83 cuvettes. They were then incubated in a 16°C water bath for 5 minutes. Next, transformation cassette DNA were added, and cuvettes were mixed gently and dried. Shock was administered at 800V and 25uF. After electroporation, transfer falcons were transported to the algae house, where they shook for 12 hours, covered by a paper towel.

Plating

Falcons were centrifuged, and 2/3 of each supernatant was discarded. The pellet was resuspended in the last ~500ul of residual supernatant, and distributed drop-wise on each plate. L-shaped spreaders were used to evenly spread the supernatant around the plate. Plates were then wrapped and placed in the algae house under low light.

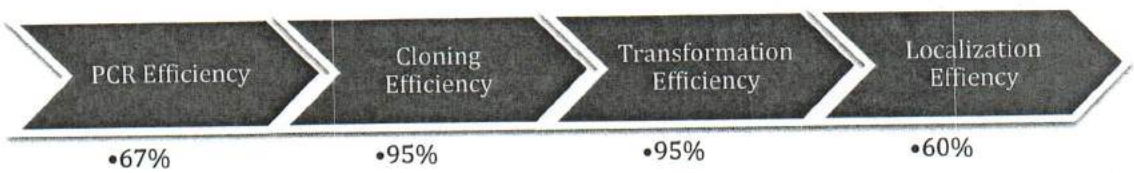
Localization

Transformation plates were screened for strong VENUS expressing colonies using the Typhoon Imaging System. Colonies were picked, and confocal imaging was done to determine subcellular localization.

Results:

Efficiency

The High Throughput Pipeline was created to efficiently tag, transform and localize photosynthetic carbon concentrating mutants. Over the course of 7 weeks, 426 DNA gene fragments were successfully amplified, yielding a 67% success rate in PCR amplification. These fragments corresponded to 243 whole genes, yielding 57% of total genes amplified. Because of shortage of time, only 83 fully amplified genes were transformed into *E.coli*, and 52 genes were localized efficiently.



Electroporation:

After electroporation was completed, the number of colonies was estimated per plate. It appeared that the length of time that the transformation cassettes were placed in the 4°C water bath affected the transformation efficiency. The samples that waited the longest had fewest transformants. A linear regression was performed in Excel to compare the amount of colonies correctly transformed by electroporation against the time that the cassettes were in the water bath (Figure 3). The R^2 values of 0.6 indicates that there was a strong linear relationship between the time of transformation and the amount of colonies.

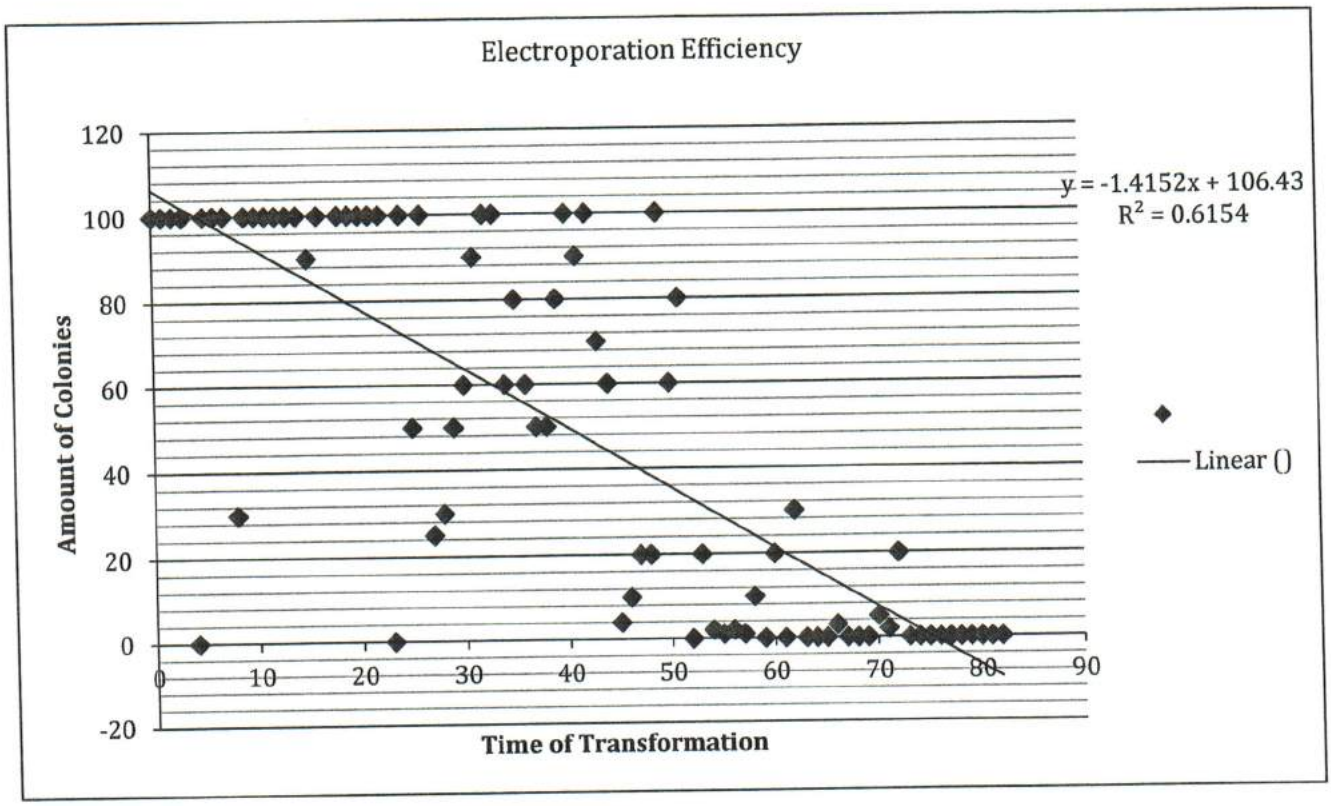
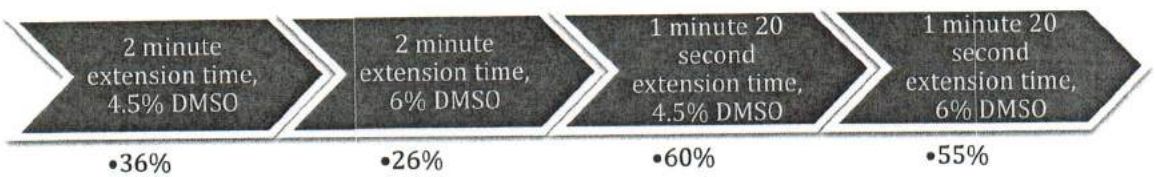


Figure 3: Electroporation Efficiency

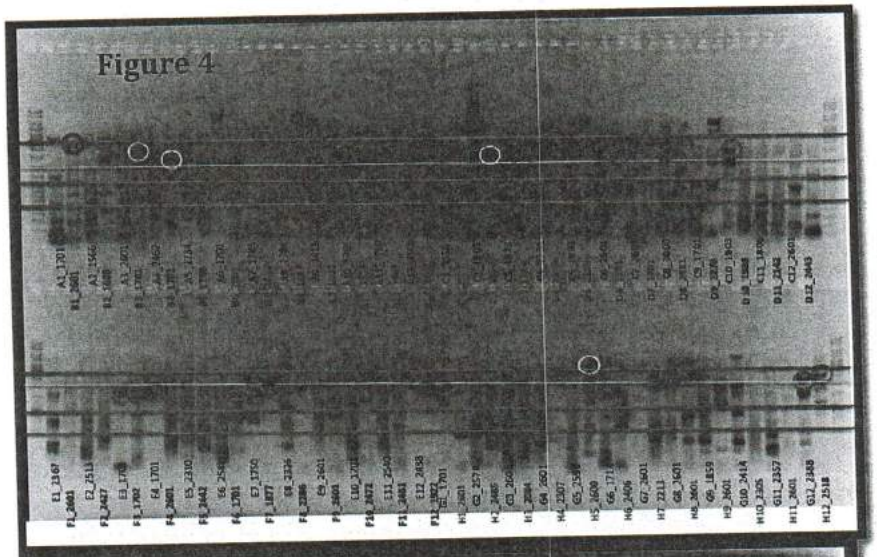
Optimization of PCR:

In attempting to optimize PCR efficiency, annealing temperatures, extension times, and DMSO concentrations were altered. Fourty-Four previously failed primers were run through 4 different PCR conditions.

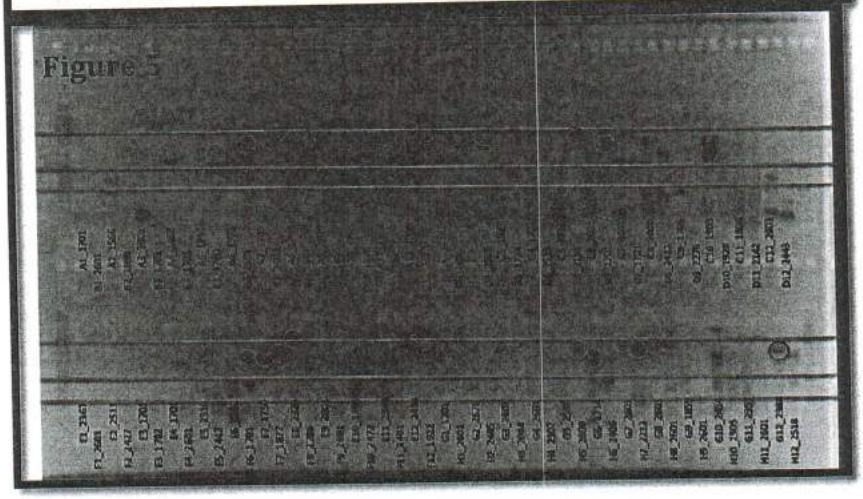


Optimization was most successful with a 60% success rate, when primers were run with a 1 minute 20 second extension time and 4.5% DMSO. Standard protocol is 6% DMSO and 30 second extension time per kilobase, where most primers averaged 3000 amplicons.

In other cases, temperature was altered to try to optimize PCR results. Figures 4 and 5 show images of two 96 well plates, with 96 separate primers. Each plate contained the exact same master mix and primers, but the annealing temperature of the program in the thermo cycler was altered from 72°C Touchdown PCR (Figure 4), to 72°C standard PCR (Figure 5).



72°C Touchdown PCR
31/96 Correct Amplicons
32% Correct



72°C Standard PCR
42/96 Correct Amplicons
44% Correct

Although the number of successful PCR reactions increased between touchdown PCR and standard, it was determined that temperature was more primer specific, and it was difficult to quantify the effect.

Localization

In total, 60% of plated genes were localized correctly. Previously, multiple known organelles were marked to aid in determining subcellular localizations, and with the identification of unknown proteins.

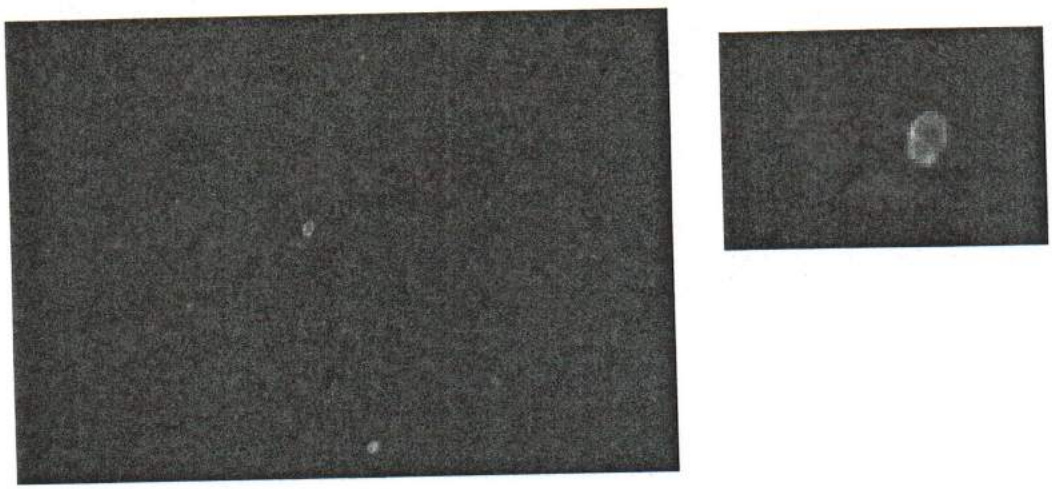


Figure 6

Figure 6 depicts the localized gene Cre04.g229300_F, a suggested RuBisCO Activase coding protein. This protein is thought to catalyze the activation of RuBisCO in vivo by removing an inhibitory sugar phosphate. The concentrated green pocket indicated that the gene was localized in the pyrenoid. In total, 83 genes were localized, and 60% were identifiable.

Conclusion:

The results from this experiment signify that a High Throughput Tagging Pipeline can be successfully utilized to amplify and localize putative photosynthetic genes of the Carbon Concentrating Mechanism in the alga *Chlamydomonas reinhardtii.* The pipeline can be optimized by recovering failed PCR primers, by increasing DMSO levels and decreasing extension times. It was also found that electroporation efficiency is negatively affected by increasing time transformation cassettes left in a 4°C water bath.

Discussion

One of the biggest challenges in studying the *C. reinhardtii* Carbon Concentrating Mechanism is identifying essential inorganic carbon transporters, and clarifying their prospective specific regulatory mechanisms. There are numerous transported proteins that are difficult to identify because single mutations do lead to an obvious change in growth phenotype. Although many CCM components are identified in cyanobacteria, they do not align with the *C. reinhardtii* genome. This broadens the possibility that the two CCM's may have evolved independently of each other, making gene identification even more difficult.

A large number of genes relating to the CCM have been discovered by various mutant screenings, but their function and interaction with the genome are still unknown. Through the development of a CCM tagging high throughput pipeline, these genes are being identified and localized at a rapid rate, revealing novel genes in the CCM. (Wang, 2010)

When utilizing this HTTP pipeline, there was a bottleneck at the PCR phase. Due to the fact that *C. reinhardtii* DNA is so rich in Guanine and Cytosine bases, PCR sensitivity increased. The pipeline yielded a 67% success rate with the many CCM genes, lower than the standard 77% for a single gene transfer. It was found that increasing DMSO and lowering extension time would optimize PCR products, and increase the Pipeline efficiency by 60%. Additionally, it was found that transformation by electroporation in *C. reinhardtii* is time sensitive. In the future, the electroporation step should be done with a smaller group of cassettes, or with more than one electroporator.

At this stage in the project, 83 genes were transformed in *C.reinhardtii*. Establishing that the RuBisCO Activase coding protein was localized in the pyrenoid indicated that it has a putative role in the CCM, and suggested that further research should be done to clarify the exact function of that gene. The role of the pyrenoid remains an important topic in CCM research. When cells are shifted from high to low CO_2 growth conditions, there is a dramatic rearrangement of starch granules. In low CO_2 conditions, a starch shell forms around the pyrenoid. Because almost all of the RuBisCO is contained within the pyrenoid, understanding aspects of the pyrenoid are essential for the creation of a viable CCM.

Because of time constraints, the other 82 genes will be imaged and analyzed at a later date. Following final optimization and image analysis, complementation vectors will be constructed with different resistance markers. An easy shuttling strategy will be devised to facilitate this process. Additionally, a database can be set up with the *C.reinhardtii* localization images. This increased availability of information to other labs will assist in all realms of *C.reinhardtii* research.

C.reinhardtii is not only used in CCM research, but in biotechnology to establish models for the low cost production of vaccines. Antigens from various pathogens including *Plasmodium falciparum*, foot and mouth disease virus, Staphylococcus, and swine fever virus, have all been produced in *C.reinhardtii*. Designing new strategies to optimize the expression of nuclear encoded target antigens will be beneficial in vaccine production. Pipelines like the High Throughput Pipeline used in this study will help increase gene localization and further the research in *C.reinhardtii* vaccine production. (Mendoza, 2013)

It is estimated that there will be about 11 billion people living on the earth by 2100. In order to provide food for this growing population, new innovations need to be made to increase food production. Transforming a viable Carbon Concentrating Mechanism into crop plants augments photosynthetic productivity by increasing levels of inorganic carbon around RuBisCO, thus decreasing photorespiration. This advance could have significant impacts on agricultural development internationally.

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