SCREEN FOR PHOTORECEPTOR AND OTHER EYESPOT DEFECTIVE MUTANTS IN CHLAMYDOMONAS

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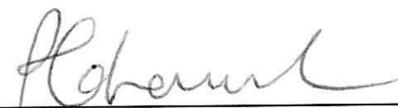

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Abstract

Chlamydomonas are single celled photosynthetic organisms that rely on their single eyespot to help them navigate towards light for use as an energy source. Their eyespot is crucial for their survival and as a result, the genetics behind the formation of the eyespot is regularly studied. To study and analyze several specific genes thought to be involved in eyespot function and formation, I collected phototaxis-defective mutants after mutagenic transformation of the *arg7-2* auxotrophic strain with the *ARG7* gene (arginine biosynthesis). Transformation of the Chlamydomonas genome was done by electroporation. The genes targeted in this study code for COP4 (photoreceptor), SOUL3 (affects eyespot size and placement), the MORN Repeat (attaches proteins to the membrane), MEC17 and MAP65 (affects tubulin dynamics), and the (PAP)-fibrillin domain (stabilizes pigment granule arrays) proteins. The *ARG7* gene inserted into the Chlamydomonas genome randomly, and I intended to screen ultimately for strains with specific disruptions in these genes. The electroporation transformants were collected and analyzed using phototaxis techniques in order to find strains containing mutations that affect the functionality of the eyespot. Phototaxis-deficient mutants will be screened by PCR to identify those with disruptions in any of the targeted genes. Fluorescence and light microscopy techniques will be used to identify the mutant phenotypes and sequencing will determine the precise location of the gene insertion.

Introduction

Chlamydomonas reinhardtii are single celled green alga that have two flagella and a single eyespot that senses light. As photosynthetic organisms, Chlamydomonas are model organisms for studying how cells respond to light. The eyespot is responsible for the ability to sense light and the cells use their flagella to phototax towards or away from light depending on the intensity (Schmidt, *et al.* 2006). The eyespot is placed asymmetrically within the organism and provides a great basis for understanding cellular asymmetry within eukaryotic organisms (See Figure 1A) (Dieckmann, 2003). In order to understand the eyespot and its asymmetric placement, it is important to understand how and in what order the eyespot is formed.

Many studies have been performed on the formation of the eyespot and it has been found that many proteins contribute to this process (See Figure 1C). It has been hypothesized that two of the key proteins in this process are Channelrhodopsin 1 and 2, ChR1 and ChR2 respectively. It has been seen that these photoreceptors move along the D4 rootlet, which determines the longitudinal placement of the eyespot (See Figure 1B) (Boyd, *et al.* 2006). This led to the hypothesis that the photoreceptors were responsible for the latitudinal placement of the eyespot. In order to test how and the way in in which the eyespot forms, from the outside-in or inside-out, a mutant strain without both photoreceptors is needed, because many of the functions of the photoreceptors are similar (Schmidt, *et al.* 2006). Currently, no double photoreceptor mutant strain exists, so an attempt to isolate this strain was made in this experiment.

Because of the low rate of homologous recombination in Chlamydomonas, directed gene knockout techniques used in yeast and other organisms are ineffective. As a result, the use of reverse genetic techniques has proven useful in the last few years (Gonzalez-Ballester, *et al.* 2011). One such technique involves the selection for transformants containing an auxotrophic

gene marker after introducing plasmid DNA by electroporation. In Chlamydomonas, the plasmid inserts in the genome randomly, and acts as an insertional mutagen. Then, the transformants are screened by PCR for the desired mutations using a primer that will only produce a product if the marker is inserted into the targeted gene (Gonzalez-Ballester, *et al.* 2011). These experiments have proven successful in the past, but in order to save time, Mark Thompson, a graduate student in the Dieckmann lab and my direct lab mentor, came up with a process to make this experimentation more efficient.

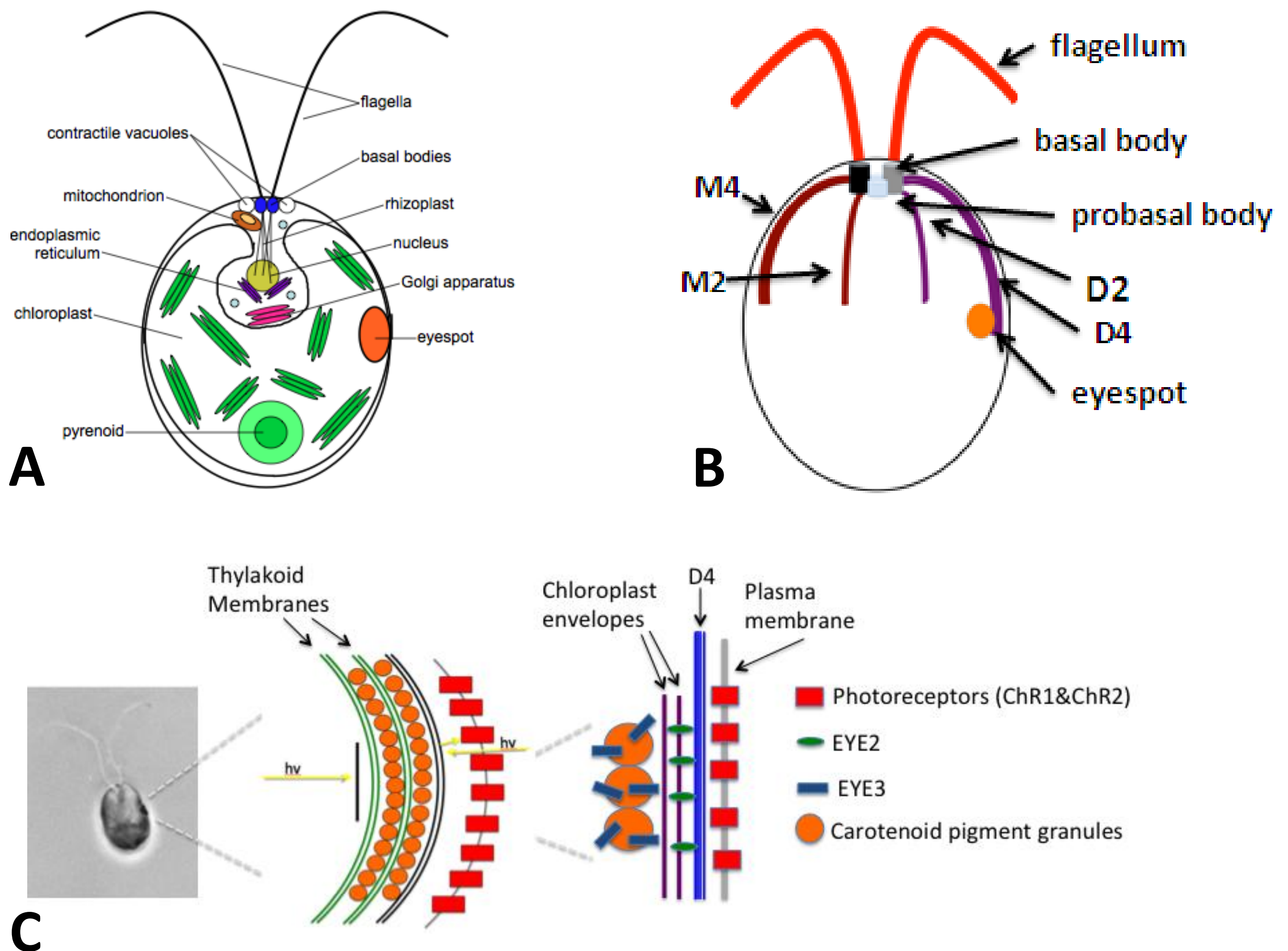
In this experiment, Mark transformed cells by electroporation with a certain marker and then I screened these transformants for the inability to phototax. Because phototaxis is a known process of a healthy and functioning eyespot, cells that do not phototax will likely have a defect with their eyespot (Dieckmann, 2003). The cells that were phototaxis-deficient were looked at by light microscopy to determine whether they had mutant eyespot phenotypes. The phototaxis-defective transformants were then analyzed using PCR which is designed only to create products if our genes of interest are interrupted. Doing this experiment in this manner allowed for a faster process that reduced the number of transformants needed to undergo PCR, in addition to reducing the number of transformant colonies needed to be maintained.

Throughout this experiment, Mark planned to target various different genes in order to better understand what proteins are necessary in eyespot formation and what their specific function is. One of these is *COP4* which codes for ChR2 (Nagel *et al.* 2003). In order to get a strain containing mutations in both photoreceptors, a strain containing a null *COP3* gene was used. *COP3* codes for ChR1 (Nagel *et al.* 2003). In addition to targeting the photoreceptor genes, the plan is to target genes that are known to code for proteins used in eyespot formation. We will also be targeting the gene that codes for the MORN repeat domain which has been shown to help

in attaching proteins to membranes (Schmidt, *et al.* 2006) and the gene that codes for the PAP-fibrillin domain which may stabilize and organize pigment granule arrays (Schmidt, *et al.* 2006). Also, the genes for the SOUL3 heme-binding protein which has been shown to affect eyespot size and location will be targeted (Schulze, *et al.* 2013). Finally, because it is known that the D4 rootlet assembly is tied to eyespot formation, the genes for the MEC17 and MAP65 proteins, which affect tubulin dynamics, will be studied (Boyd *et al. 2011).*

As far as my role in this experiment goes, I was responsible for the isolation and maintenance of the transformant colonies in the 96-well plates in addition to screening them for phototaxis-deficiencies. The hope was to identify transformants that have mutations affecting their eyespots and use PCR techniques to determine what genes are interrupted and their overall function in relation to the eyespot. So far, a total of about 6,800 transformants have been analyzed and 36 phototaxis-deficient transformants have been found. PCR has been performed on about half of these transformants and no mutations within the target genes have been identified yet.

Figure 1: Structures of Chlamydomonas cells



A. Depicts key features and asymmetry of the eyespot in the Chlamydomonas cell (courtesy of Mark Thompson. Boyd *et. al.* 2011).

B. Shows the asymmetrical location of the eyespot and the rootlets forming from the basal bodies. The longer D4 rootlet is depicted and the eyespot forms towards the bottom of the rootlet at the equator of the cell (courtesy of Mark Thompson).

C. A closer look at the eyespot structure which shows the two main compartments in the cell, the chloroplast and the plasma membrane. EYE3 marks the pigment granules,

EYE2 localizes to the chloroplast envelope and the photoreceptors, ChR1/2 localize to the plasma (courtesy of Mark Thompson).

Materials and Methods

Media and Strains of Chlamydomonas Used

Two media were used during this experiment. The first was Tris-acetate phosphate (TAP) medium (Tris, TAP salts, phosphate solution, trace metals, glacial acetic acid, water) (Gorman & Levine 1965). The second medium was minimal (M) (Tris, TAP salts, phosphate solution, trace metals, water) (Gorman & Levine 1965). Liquid forms of both TAP and M media were used and solid agar TAP medium was used. One *Chlamydomonas reinhardtii* strain used contained a null *ΔCOP3* gene and the *arg7-2* mutation and the other had just the *arg7-2* auxotrophy.

Electroporation and Selection for Transformants

On the first day, cells were inoculated in 3 mL of liquid TAP medium in a test tube. When the cells were re-suspended, they were transferred to a flask containing 250-300 mL of liquid TAP medium. A hemacytometer was used to count the cell density. A cell density of between $1x10^6$ and $5x10^6$ cells/mL was desired and the cultures usually reached this density within 2-3 days. Once the cells were of an appropriate density, they were added to a 250mL bottle and centrifuged at 4000 rpm for ten minutes. The supernatant was removed and the cells were re-suspended using 20 mL TAP medium and 50 mM sucrose in a 50 mL sterile tube. The cells were then incubated in light for 2-4 hours. The cells were collected again at 4000 rpm for 10 minutes and re-suspended using the same solution (TAP and Sucrose) to a final concentration of ~$3.5x10^8$ cells/mL. Next, 10 µL of the transforming DNA was put in a 1.5 mL glass test tube. Then, add 300 µL of the cell suspension was added to the tube and mixed by gentle pipetting. This mixture was transferred to a pre-chilled electroporation cuvette on ice and left there for 10-

20 minutes. The BioRad Genepulser Xcel was used to electroporate. It was set to a capacitance of 25 μF, a voltage of 0.8 KV and a resistance of 1000 Ω. After the cells were shocked, they were left at room temperature for 5-20 minutes and then transferred to 10 mL of liquid TAP medium in a tube. They were then slowly shaken for ~12-16 hours. The cells were then decanted into a 15 mL tube and centrifuged at 4000 rpm for 5 minutes. They were re-suspended in ~300-750 μL of liquid TAP medium by gentle pipetting so that ~150-200 μL of cells were put on each plate. The cells were then spread on 2-3 plates and transformant colonies usually appeared after 4-5 days. This procedure was performed and adapted by Mark Thompson from Dr. Brad Olson's lab protocol at Kansas State University. The parameters for the electroporation procedure were taken from Dr. Patrice Hamel's lab protocol at Ohio State University.

Picking of Transformants

Using toothpicks, Arg+ transformant colonies were transferred from the solid agar TAP medium into 96-well plates that were filled with 200 μL of liquid TAP medium in each well (Gonzalez-Ballester *et al.* 2011). One colony was picked at a time and put into each well. Care was taken to never put two colonies in one well. These plates were wrapped with parafilm to prevent evaporation and were grown for 2-3 days in the incubator.

Phototaxis

150 μL of each colony from the 96-well plate was transferred using a micropipette into corresponding labeled test tubes containing 2 mL of M medium each. These tubes were then grown in the incubator overnight. The next day, phototaxis analysis was performed on each of the samples. To do this, the test tubes were placed upright in a covered box with a small slit cut

at the bottom. This slit was placed towards a light source and the box was wrapped in tinfoil to ensure that the only light let into this box was through this slit. The test tubes were left in there for at least fifteen minutes. They were taken out at this time and analyzed to see if the cells could swim and if they could phototax. If they could phototax, the bottom of the tube would have a high concentration of cells and be dark green. If they could swim, the tubes would return to a uniform green color after being left out in the light for a few minutes.

Pooling and PCR Analysis of Colonies

For the first trial, every 96-well plate that was filled with transformant colonies was analyzed using PCR techniques. To do this, 40 μL of each colony from each well on one plate was transferred using a micropipette into a larger flask. This sample was then used for PCR screening (Gonzalez-Ballester *et al.* 2011). For the second trial, pooling did not occur. Individual colonies which do not phototax were spun down, re-concentrated, and then plated on solid agar TAP medium plates. These colonies were grown, reanalyzed for phototaxis and then PCR will performed on them.

Results

The total number of transformants picked and collected for the first trial totaled roughly 10,000. These filled 105 plates of 96-wells each. Of these 10,000 transformants, 2,500 were analyzed for an inability to phototax or swim. Twenty phototaxis-deficient transformants were identified and 192 transformants could not swim.

The twenty phototaxis-deficient samples we found were phenotypically analyzed by light microscopy. No notable eyespot mutant phenotypes were seen. The cells were regrown in liquid TAP medium and a phototaxis assay was performed again. The cells were not phototaxis-deficient in this second trial. Additionally, PCR was performed on the cells. The result did not show any interesting gene mutations.

Number of Mutants Screened	Number of Phototaxis-deficient	Number of Swimming Negative	Percent of Phototaxis-deficient	Percent of Swimming Negative
2500	20	192	0.8%	7.68%

Table 1: The number of transformants screened, the number of phototaxis and swimming deficient strains and the percentage of them in relation to the total samples analyzed.

Many of these transformants died before analysis could occur. In addition, many gave false phototaxis information. This was due to too large of an electroporation sample. The second trial was smaller in order to reduce the amount of dead and incorrect samples created.

A second electroporation was performed and a second set of transformants was picked and analyzed. In this trial, 4,320 transformants were analyzed, which filled 45 plates with 96-wells each. Of these, it was seen that sixteen did not phototax and ten did not have the ability to

swim. For this second set of transformants, PCR has not been performed on the phototaxis-deficient transformants yet. Light microscopy analysis has been done on a few samples and only one sample has been observed to have an abnormal phenotype. This sample exhibits larger than normal eyespots.

Number of Mutants Screened	Number of Phototaxis-deficient	Number of Swimming Negative	Percent of Phototaxis-deficient	Percent of Swimming Negative
4320	16	10	0.37%	0.23%

Table 2: The number of transformants screened, the number of phototaxis and swimming deficient strains and the percentage of them in relation to the total samples analyzed.

Discussion

In this experiment, electroporation to generate transformants has been successfully completed. Two trials have been run so far. In the first trial, over 10,000 transformants were isolated all at once. As a result, there was not enough manpower to follow through with the rest of the experiment. The transformants were picked and left to grow in the 96-well plates for well over three days. Phototaxis analysis was then performed on them and many transformants were found to be phototaxis-deficient and an even larger number were found to be unable to swim.

When light microscopy was performed, no abnormal phenotypes were observed. Additionally, when phototaxis analysis was performed a second time, with a shorter incubation time, all the transformants performed phototaxis properly. Each 96-well plate was pooled and PCR was performed on these samples. No products were created which meant that none of our target genes had been interrupted during the electroporation.

Through this first trial, it became clear that this method of experimentation was not working. First, it was obvious that less transformants needed to be made at one time. By having this many transformant colonies, phototaxis analysis could not be performed in a reasonable time frame and many of the samples were left in liquid for far too long before analyzed. This allowed them to get old, and lose flagellar mobility which accounted for the inability of many of the samples to swim in addition to the false phototaxis-deficient results we found. Also, all the transformants that were picked were not even analyzed because they dried out and died before we could test them. In addition, it became clear that doing PCR on all of the samples was a waste of time because the results we were looking for would only be obtained from a phototaxis-deficient transformant anyways.

For the second trial, some adjustments were made. First, smaller electroporation cycles were performed. For each cycle, about twenty 96-well plates were done at a time as opposed to a hundred plates. The electroporation was performed every few weeks which allowed for a continuous cycle between picking transformants, incubating them and performing phototaxis analysis on them. No sample was allowed to sit for longer than two days before being analyzed. As a result, many of the transformant colonies observed have not lost their ability to swim and fewer phototaxis-deficient cells were found. So far, sixteen phototaxis-deficient cells have been observed. Light microscopy has not been performed on all of these samples, but for the ones it has been, no noticeable eyespot mutant phenotypes have been observed. Next, these phototaxis-deficient samples will be centrifuged and re-concentrated. They then will be plated on solid TAP media plates, grown, and PCR analysis will be performed on them.

The hope is that one of these samples will create a product when PCR analysis is performed. When this happens, one of the genes in question will be mutated and we will have successfully identified this mutation. From there, Mark plans on characterizing the mutants by fluorescence microscopy with antibodies to known eyespot components, and analyzing combination of these new gene mutations with previously characterized eyespot mutations.

Through my work on this project, I have successfully learned how to perform a large-scale genetic screen and how to adapt when problems arose. It became clear that this project was originally much too large for us to execute properly and I learned how to successfully modify the project so that it functioned properly for the amount of manpower present. This allowed us to come up with a suitable protocol that allowed us to advance towards our final goal.

Upon completion of this project, the formation of the eyespot in Chlamydomonas will be better understood. An understanding of what proteins are present as well as what their function

is will be formed. The new knowledge obtained from this experiment will hopefully be expanded to other eukaryotic organisms and help to better understand how asymmetric cellular development and complex assemblies involving multiple membranes occurs.

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