ISOLATION AND CHARACTERIZATION MUTANTS DEFECTIVE IN CILIA REGENERATION IN *CHLAMYDOMONAS reindhartii*

Honors Thesis

Presented in Partial Fulfillment of the Requirements
For the Degree of Bachelor of Science in Biology

In the College of Arts and Sciences
At Salem State University

By

Ellen Acheampong

Dr. Jason M. Brown, Ph. D.
Faculty Advisor
Department of Biology

Commonwealth Honors Program
Salem State University
2019

ABSTRACT:

Cilia and flagella are identical brush-like organelles found on the surfaces of eukaryotic cells and are involved in motility, sensing, and signaling. Defects in cilia assembly or function lead to polycystic kidney disease, congenital heart disease, Bardet Biedl syndrome, and other emerging ciliopathies. In *Chlamydomonas reinhardtii*, one of the most well-studied flagella model organisms, regeneration of flagella to normal length and normal function occurs within 90 minutes of acid-shock deflagellation. During this process hundreds of genes are induced. Much is left to be determined about how cells regulate expression of these genes: How do cells detect the presence or absence of cilia? How do cells send an 'absence of cilia' signal to the nucleus? How are the hundreds of genes encoding cilia proteins coordinated with each other? Previous experiments found that cells that failed to upregulate a reporter of flagella gene expression also had a delay in flagella regeneration.

The goals of this experiment were to generate new *C. reinhardtii* insertional mutants defective in cilia regeneration, to identify the mutated genes in these strains, and ultimately, to identify transcription factors and signaling components needed for flagella assembly. 3000 hygromycin-resistant colonies were generated by the insertion of *aph7"* DNA fragment through electroporation. 42 of the 3000 colonies exhibited defective flagella structure, defective motility, or delayed flagella regeneration; 14 of these 42 mutants had delay in regenerating their flagella. To identify the mutated genes, genomic DNA for a subset of mutant strains was extracted, and Polymerase Chain Reaction (PCR) reactions were set up using the Restriction Enzyme Site Directed Amplification polymerase chain reaction (RESDA PCR) protocol with primers specific for the a*ph7"* insert

combined with four different degenerate primers. DNA fragments from successful PCR reactions were gel purified and sequenced. Using this method, mutated genes were identified and characterized to identify the proteins encoded by these genes. Flagella regeneration and reporter gene assays were conducted to further characterize the phenotypes of two of the mutants originally identified as having a delay.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

Dr. Jason M. Brown, Ph. D. - Thesis Advisor, Professor of Biology

Kathy Murphy ’75 – SSU Trustee

Dr. Thomas Nelson Scottgale – Academic Advisor

Dr. Scott Nowka – Honors Program Chair

Dr. Ryan Fisher – Department Chair

Dr. Lynn Atkinson – Professor of Biology

Pamela Cortes-Ortiz – Graduate Resident Assistant

Commonwealth Honors Program

Salem State University

Biology Department

Team Cilia

Irune Aparicio

Funding Sources:

The Kathy Murphy Summer Research Academy in Biology Awards.

The Alfred L. Borgatti Biology Scholarship.

LIST OF TABLES AND FIGURES

INTRODUCTION:

Cilia and flagella are identical brush-like organelles found on the surfaces of eukaryotic cells and are involved in motility, sensing, and signaling. Flagella and cilia are functional in diverse cell types: the beating of cilia in the bronchi of the lungs keeps airways clear of mucus and debris; the flagellum of a sperm cell propels the cell to the egg and is an essential step in the life cycle of humans and most complex organisms; and cilia and flagella are used for feeding and reproduction in organisms as diverse as clams and algae (Lindemann and Lesich, 2010). Defects in cilia assembly or function lead to respiratory infections, male infertility, situs inversus - a rare anomaly in which transposition of the organ to the opposite side of the body occurs (Pitiakoudis et al. 2005), polycystic kidney disease, retinal degeneration, and Bardet-Biedl Syndrome (Albee, et al. 2013), congenital heart disease, and other emerging ciliopathies. Primary Ciliary Dyskinesia is also known as immotile cilia syndrome because it is caused by defects in motile cilia. Symptoms include chronic bronchitis and sinusitis since the cilia are unable to clear mucus and inhaled bacteria from airways. Frequent bacterial infections can lead to bronchiectasis (Brown, et al. 2017). Bronchiectasis is a chronic lung disease with permanent airway dilatation, mucus retention and recurrent lower respiratory tract infections (McCallion and De Soyza, 2017). Deficiency of the regulatory factors of cilia can also cause problems like alteration of the composition of the hormone- secreting cells of the islet of Langerhans, defects in the ciliary growth and functions (Brown, et al. 2017).

There are two types of cilia; motile and non-motile cilia. The motile cilia use their brush-like structures to help move mucus and foreign particles in the body. Motile cilia also contain additional protein complexes that are essential for motility, such as the nex-

in-dynein regulatory complex (N-DRC), which regulate the activity of the dynein arms (Choksi, et al. 2014). The non-motile cilia play a crucial role in many different organs such as serving as antennae that receive sensory information for the cell and processing signals from the other cells or the fluids surrounding it (Kong, et al. 2015).

All cilia share a common underlying morphology; however, cilia vary extensively in their shapes and sizes, ultrastructural details, number per cells, motility pattern, and sensory capabilities. The internal cytoskeletal arrangement of flagella and cilia are composed of other protein complexes associated with the cylinder of 9 doublets which collectively compose the axoneme (Lindemann and Lesich, 2010). Some cilia have 9+0 microtubule configuration whereas others have 9+2 microtubule configuration where there is a central pair (CP) of singlet microtubules (Choksi, et al. 2014). The 9+0 versus 9+2 configuration usually distinguishes the non-motile from the motile cilia, respectively. Each of the nine doublets has a protein arm-like projection that provides the motive force for the flagellar to beat (Gibbons and Rowe, 1965). The protein was identified as dynein in 1965 by Gibbons and Rowe (1965). However, the dynamics of the dynein protein is not yet fully understood (Lindemann and Lesich, 2010). At the base of cilia lie microtubule-based structures called basal bodies, which template and anchor cilia and recruit proteins needed for ciliary assembly (Albee, et al. 2013). The presence of cilia and basal bodies is linked to the cell cycle in many organisms; mammalian cells that exit the cell cycle are quiescent, nonproliferating, and assemble cilia (Tucker, et al. 1979).

Interest in cilia as an important organelle has increased due to a growing list of human diseases associated with ciliary defects, which cause a wide range of phenotypes that include renal cysts, liver disease, cognitive impairment, retinal degeneration, obesity,

skeletal bone defects, laterality defects, and polydactyly (Albee, et al. 2013). Abnormal formation or function or defects in the configurations of the microtubules can cause any of the above-mentioned cilia dysfunctions. Also, abnormal formation of cilia has been implicated as an underlying cause of many syndromes and disorders that have traditionally been recognized as disjoint conditions (Albee, et al. 2013). The identification, characterization, and implication of human ciliopathy disease genes have greatly benefited from study in the model organism *C. reinhardtii* (Pazour, et al. 2005).

When it comes to studying flagellar motility, *C. reinhardtii* is an ideal model to use. It is widely used in genetics, biochemistry, molecular biology, microscopy, etc. because of its rapid growth rate in a short period of time with a little amount of money. Also, *C. reinhardtii* can be easily mutated with a selectable gene. There are also human-related studies that can be done with *C. reinhardtii* cells to generate results that help scientists to study issues associated human health. *C. reinhardtii* is also a model organism widely used in the study of chloroplast biology, photosynthesis, and lipid metabolism for biodiesel production (Meslet-Cladiere and Vallon, 2014). Also, microscopy can be done with *C. reinhardtii* cells to get beautiful images of the cells and their flagella.

A study by Dentler and Rosenbaum (1977), indicated that flagellar elongation occurred by the distal addition of subunits. Most flagellar microtubule assembly during flagellar regeneration *in vivo* occurs by addition of subunits to the distal tip, and *in vitro* assembly studies show that brain tubulin assembles primarily onto the distal end of the central and outer doublet microtubules (Dentler and LeCluyse, 1982). The distal ends of the central microtubules insert into the central microtubule cap which is attached to the distal tip and remains attached during flagellar regeneration and resorption. According to the

study, the central microtubule caps were present before deflagellation, during and after regeneration. They suggested that the central microtubule caps must have been one of the first flagellar structures to be assembled. However, Dentler and Rosenbaum (1977) observed in the same study that the central microtubule caps were blocking the addition of brain tubulin subunits onto the distal tip of the microtubules. This suggested that the CP microtubules might assemble by the addition of subunits to their proximal ends due the presence of the central microtubule caps (Dentler and Rosenbaum, 1977). However, a more recent experiment by Lechtreck, et al. (2013) showed that the CP self-assembles without requiring a template at either the transition zone or the flagellar tip. This refutes earlier suggestions that the CP might assemble with addition to the proximal ends.

In *C. reinhardtii*, one of the most well-studied cilia model organisms, regeneration of flagella to normal length and normal function occurs within 90 minutes of acid-shock deflagellation. There have been studies on the functions of cilia in *C. reinhardtii* over the past 50 years; these studies have shown that the functioning of cilia and their development are regulated by IFT, a bidirectional process in which proteins move within the cilia – from the cell body out to the ciliary tip and back. During this process hundreds of genes are induced. Much is left to be determined about how cells regulate expression of these genes. Some questions that prompted this experiment were: How do cells detect the presence or absence of cilia? How do cells send an ‘absence of cilia’ signal to the nucleus? How are the hundreds of genes encoding cilia proteins coordinated with each other? By doing this experiment, we hoped to answer the question of what regulates the ciliary functions of *C. reinhardtii*, and which unknown transcription factors regulate the regeneration of the flagella after deflagellation.

Genes encoding some of the IFT proteins have been shown to be regulated by regulatory factor X (RFX) transcription factors (TFs). An example is *daf-19* of *C. elegans* which is regulated by RFX TF. Experimental evidence has shown that RFX factors are intrinsically tied to the transcriptional regulation of ciliary genes stemming from work performed in *C. elegans* (Choski, et al. 2014). However, *C. reinhardtii* does not have RFX TF. RFX family proteins share a highly conserved winged-helix DNA-binding domain and are required for the transcriptional regulation of ciliary genes in primary cilia (Swoboda, et al. 2000).

Another family of TF, FOXJ1 is believed to have co-existed with RFX in some organisms, but the expression of RFX overshadows the expression of FOXJI TF. Hence RFX is the major TF in most organisms. RFX factors appear to be required to make both motile and immotile cilia, whereas FOXJ1 is required specifically to make the motile cilia. These two transcription factors function together in cells that make motile cilia. RFX and FOXJ1 transcriptional programs interface. It appears that RFX factors regulate core cilia genes on their own and cooperate with FOXJ1 to regulate motility genes in specific cell types. Although RFX and FOXJ1 are key regulators of ciliary gene expression in animals (Choksi, et al. 2014), *C. reinhardtii* does not contain any of the orthologs of RFX, FOXJ1, or other transcription regulators known to modulate the transcription of genes encoding ciliary proteins in other organisms, indicating that the transcriptional control of the ciliogenesis is fundamentally different in unicellular and multicellular organisms (Li, et al. 2018).

The discovery of a role for XAP5 in transcriptional regulation is the first clue about the different mechanism(s) in *C. reinhardtii* and or other single-celled organisms.

XAP5 is an evolutionarily conserved protein family across diverse organisms that has recently been shown to have roles in flagellar assembly in *C. reinhardtii* (Li, et al. 2018). XAP5 proteins are present in both ciliated and non-ciliated organisms (Martin-Tryon and Harmer, 2008). The results of a study done by Li, et al. (2018) showed that XAP5 functions as a transcription factor to regulate flagellar assembly in *C. reinhardtii*. In the same study, the researchers also tried to understand the role XAP5 plays in flagellar assembly by examining XAP5's expression and subcellular localization. Their findings included that XAP5 was localized in the nucleus and the appropriate nuclear localization of XAP5 mediated by the nuclear-localization signal was required for flagellar assembly. Also, the expression and subcellular location of XAP5 had no significant change during flagellar regeneration. However, a posttranslational modification in XAP5 was reflected by a change in its electrophoretic mobility in their experiment. This occurred when XAP5 was modified immediately after deflagellation, and the modification of XAP5 disappeared as the flagella reached nearly their full length. XAP5 was not modified after lysates of the mutant strains were incubated with a phosphatase suggesting that XAP5 was phosphorylated during flagellar regeneration (Li, et al. 2018).

The data from their study suggested that the transcriptional regulation of flagellar genes might be classified into two types: XAP5-dependent and XAP5-independent. The transcriptional control of more than 100 flagellar genes was XAP5 dependent in the experiment done by Li, et al. (2018), whereas the expression of several flagellar genes was XAP5 independent, implying that the regulation of flagellar genes at the transcriptional level is a highly complex process and that XAP5-independent flagellar gene expression can be regulated by other, undiscovered transcriptional mechanisms.

An important gene that also plays a role in the functions of the flagella in *C. reinhardtii* is the *LC8* gene. LC8 is a small, yet vital, protein in a wide spectrum of protein complexes which functions as a dimer with two identical grooves formed at the dimeric interface (Gupta, et al. 2012). Structural studies of dynein intermediate chains (ICs) suggest that the two grooves of an LC8 dimer bind both chains in a dimer to enhance the stability of a molecular complex (Williams et al. 2007). LC8 is present in axonemal and cytoplasmic dyneins and the radial spoke (RS) complex in eukaryotic cilia and flagella (King and Patel-King, 1995). The radial spoke in *C. reinhardtii* comprises of LC8, RSP3, and at least 17 other distinct RSPs that are critical for the beating of the flagella. The severe deficiencies of the RS in *LC8* mutants suggest that LC8 plays a critical role in this complicated assembly process (Gupta, et al. 2012). In the same study by Gupta, et al. (2012), they found that LC8 is tightly linked to RSP3 phosphorylation and RSP3 is an LC8-binding protein in the RS, and LC8 binding affects RSP3 and RS assembly in multiple related events. These results shed light on the process of ciliogenesis and the diverse roles of LC8 on LC8-containing complexes. In *C. reinhardtii, LC8* gene gets upregulated when the cells lose their flagella. But how *LC8* regulation is associated with the regeneration of the flagella is still unknown. Could the upregulation of the *LC8* gene in the absence of flagella be one of the undiscovered XAP5-dependent or independent transcriptional mechanisms described by Li, et al. (2018)?

LC8 promoter and terminator fused to the *Gaussia* luciferase coding sequence was used to create an *LC8* construct which measured the upregulation of luciferase in *C. reinhardtii* in this experiment. In an unpublished result from a previous experiment (Brown and Witman, in preparation), two mutants with an inability to upregulate the *LC8*

reporter were identified. These mutants had a delay in regenerating flagella which suggested the approach of just screening for mutants with a delay in regenerating and characterizing the ability to upregulate the *LC8* construct or not.

The main purpose of this experiment was to generate mutants that had delays in regenerating their flagella after deflagellation, identity the proteins encoded by the mutated gene in the *C. reinhardtii* genome and identify how the nuclei sense the absence of flagella for gene induction process to begin. The experiment was done using insertional mutagenesis and transformation. Transformation can also be used to put cloned genes back into organisms to complement mutations.

Insertional mutagenesis allowed us to create mutations in the *C. reinhardtii* genome and characterize the mutated genes and the proteins encoded by the mutated genes. Insertional mutants are created by transforming cells with DNA- containing selectable makers, and insertional mutagenesis has developed a powerful way for scientists to approach genetic analysis in C. *reinhardtii* (Pazour, Witman, et al. 1998). Electroporation is the process used to open pores in the membranes of the cells to insert the desired gene into the cells. The introduced DNA inserts randomly in the genome. According to Witman, et al. (1998), insertional mutagenesis can sometimes delete a larger gene than typical. This could mean that one or more genes are deleted. Or, whole gene could be deleted (this is rare because selectable markers just insert with no deletion of the genomic DNA) which would lead to the disruption of the component and the configuration of the flagella and their functioning. This leads to difficulties in doing backcrossing and rescuing the mutated gene. It also becomes difficult to identify or prove that phenotype is caused by the disruption of the gene of interest and not an adjoining gene (Pazour and Witman,

2000). If something like this occurs, it should be certain that the mutation or the insertion is what caused the phenotype of the mutant. If not, rescuing and backcrossing experiments would not be successful (Pazour and Witman, 2000).

In this experiment, a*ph7"* gene was extracted from bacteria that contained a plasmid DNA, *pHyg3* that confers resistance to hygromycin B (Berthold, et al. 2002) by growing and cutting the plasmid with the restriction enzyme, *HindIII*. The gene was then transformed into *C. reinhardtii* cells through electroporation, and the transformants were grown on TAP (Gorman and Levine, 1965) plate with hygromycin B. Hygromycin B is an atypical aminoglycoside antibiotic with unique structural and functional properties. It inhibits protein synthesis by the ribosome (Borovinskaya, et al. 2008). Cells that were resistant to the hygromycin B drug picked up DNA and survived because the a*ph7"* gene made them resistant to the drug. And cells that were not resistant died. This was also evident in Brown, et al. (2017).

This experiment generated 3000 colonies of hygromycin-resistant transformants. The colonies of cells were picked individually and grown in growth media where they were screened for defective flagella and delay in regenerating the flagella. In Brown, et al. (2015), when both wild type cells and mutants are deflagellated, wild types cells regenerated their flagella within 90mins after deflagellation, but the mutants took longer time to initially regenerate their flagella back to the full length and functions. In total, 42 *C. reinhardtii* mutants were generated with 14 of the mutants expressing delay and 28 expressing other flagella defects. Some of the defects were palmelloid, where the daughter cells failed to detach from the mother cell wall after cell division (Li, L, et al. 2018), and funky swimming different from the wild type cells (Brown, et al. 2017).

RESDA PCR was used to amplify the extracted and purified DNA from the *C. reinhardtii* mutant strains using four degenerate primers (*Alu*I, *Pst*I, *Sac*II, and *Taq*I) and an upstream and downstream primers. The RESDA PCR reaction is based on the random distribution of frequent restriction sites in a genome and on a special design of primers. Specific primers (upstream and downstream primers) of the marker DNA combined with the degenerate primers allow amplification of DNA fragments adjacent to the marker insertion by using two rounds of either short or long cycling procedures (Gonzalez-Ballester, et al. 2005). This was two nested reactions; primary reaction and secondary reaction, allowing us to get specific bands at the end. By doing this combination, each mutant strain had 8 reactions for both the primary and secondary reactions. DNA fragments amplified using RESDA were sequenced to identify the sites of insertion and the phenotypes of two delay mutants were further characterized.

MATERIALS AND METHODS:

- GENERATION OF MUTANTS AND SCREENING OF MUTANT PHENOTYPES:

PHyg3 plasmid was isolated from bacteria QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD), and a DNA fragment, *aph7"* which confers resistance to hygromycin B (Berthold, et al. 2002) was cut out from the plasmid using the restriction enzyme, *HindIII*.

An agarose gel was used to separate the plasmid from *aph7"* through electrophoresis. A razor blade was then used to cut the 1.7 kb *aph7"* fragment out of the gel. The DNA was purified from the gel slices using Qiaex II, a purification kit from Qiagen Company (Germantown, MD).

The *aph7"* fragment was then transformed into *C. reinhardtii* using electroporation (BTX Electro Cell Manipulator 600 at wave pulse of 200V and a pulse length of 11msec) -- a process used to briefly open the pores in the cell membrane of *C. reinhardtii* using electricity to create the mutants using previously described methods by Brown, et al. (2012).

The cells (*C. reinhardtii*) were plated on agar plates (petri dishes that contained a growth medium – TAP Medium (Gorman and Levine, 1965) + 1.5% agar and 10µg/ml hygromycin B) (Berthold, et al. 2002).

Some cells picked up the DNA (the cells that survive the drug, hygromycin B), and some did not (the cells that died). Colonies of cells that survived were picked individually by hand with toothpicks and cultured in a 96-well plate with liquid M Medium (Witman, 1986). The mutant cells were deflagellated by acid shocking them and screened for delay in regenerating their flagella back to the normal length and functions. D10 wild-type *C. reinhardtii* cells with *LC8 - Gaussia* luciferase constructs were used as positive control for this experiment. The *LC8 - Gaussia* luciferase construct was designed to measure gene expression during flagellar assembly (Brown and Witman, in preparation). 160µl of growth media (M- media) were pipetted in a 96-well plate. Colonies of cells were then picked individually into the wells. The cells were grown for 1-2 days, then 10µl was transferred to backup plates leaving 150µl of cells for the deflagellation experiment. 1ml of 0.5M acetic acid was added to 16.6ml M Medium (Witman, 1986) to dilute it to 0.03M acetic acid. A liquid handler (semi – auto pipetting system), BenchSmart 96 (Metter-Toledo, Columbus, Ohio) was used to dispense 50µl of the diluted 0.03M acetic acid into the wells containing the cells. The purpose of the acid was to stimulate the cells

to voluntarily excise their flagella. The plates were then screened under an inverted microscope to make sure that the cells were well deflagellated. Immediately after 2 minutes, 50µl of 0.03M M KOH diluted from 0.5M KOH by adding 16.6ml of M Medium (Witman, 1986) to 1ml of 0.5M KOH, was quickly added to the 96-well plate using the handler to bring the pH back to 7. The mutants' phenotypes were screened prior to deflagellation. The cells were screened over a period of approximately 90 minutes for delay mutants (FIG 4).

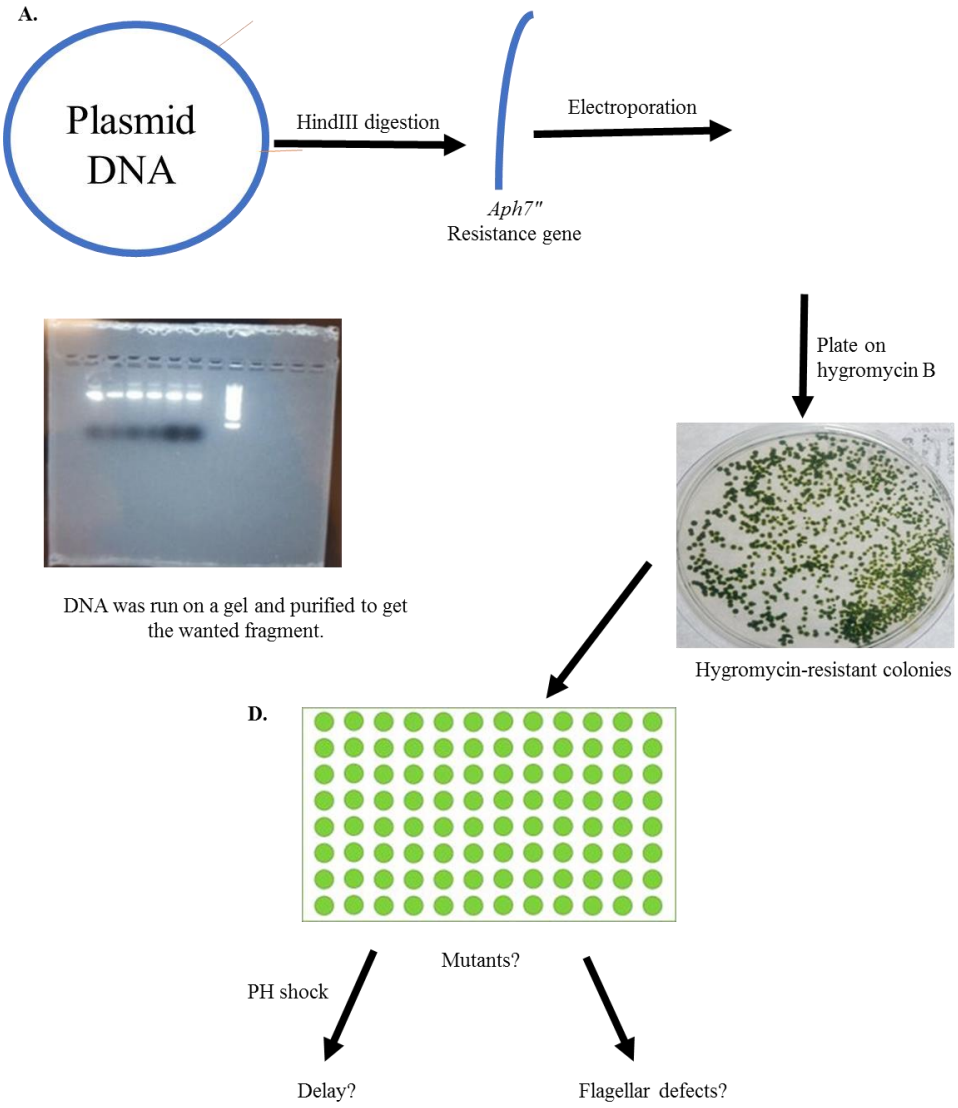

FIG 4: The *C. reinhardtii* mutants were generated by insertional mutagenesis. (**A).** The a*ph7"* gene which confers resistance to hygromycin B drug was cut out from pHyg3 plasmid DNA using HindIII restriction enzyme. The resistance gene was then purified and inserted into the cells through electroporation. **(B).** Plasmid DNA mini preps were checked on a gel prior to digestion with HindIII restriction enzyme. **(C).** The new transformants were plated on agar plates containing hygroycin B for selection. The cells that incorporated the DNA (resistance gene) were resistant to the hygromycin drug and survived. **(D).** The mutant cells were individually hand-picked into 96-well plate with growth media. In the 96-wells, the cells were induced to lose their flagella by dropping the pH of the growth media. The mutants were then screened for delay in regenerating their flagella and flagella defects.

- CHARACTERIZATION OF MUTANTS:

The genomic DNA of the mutant strain was extracted using phenol chloroform and purified. The extracted DNA samples were used for the RESDA PCR reactions (FIG 5)

RESDA PCR protocol was used to locate the insertion site in the genome and amplify the insertion site of the *aph7"* gene. Primary RESDA PCR reactions were set up with 2 different primers and 4 different degenerate primers together with genomic DNA of the mutants (FIG 5). The secondary RESDA PCR reaction on the other hand were set up with either the upstream primer and Q0 or the downstream primer and Q0 together with the primary reactions as templates. Degenerate primers have flexibility in the way they bind to the genomic DNA, which makes it easier for them to land near the specific site of the mutation. For the 1° RESDA PCR, each strain was exposed to both upstream primer and downstream primers and four different types of degenerate primers; *Alu*I, *Pst*I, SacII, *Taq*I (Gonzalez-Ballester. et al. 2005). For the 2° reaction, both the upstream and downstream secondary RESDA PCR primers were added to the reactions for each strain. A 1% agarose gel was used to run and analyze the reactions by electrophoresis. This is because

the RESDA PCR is a nested reaction. Therefore, each of the primary reactions for each strain was used as s templates for the secondary reactions. Therefore, there were no degenerate primers used in the secondary reactions. Instead, Q0 sequence was used to increase the specificities of the bands. According to Gonzalez-Ballester, et al. (2005) it was not necessary to use the degenerated primers a second time, thereby avoiding the generation of more nonspecific PCR products. Using the Q0 also had the advantage of working with a high annealing temperature (60 °C) during the second round, further enhancing the specificity of the PCR products. Each strain had all their 1° reactions diluted and used for the 2° reactions (1:50 dilution) with the secondary upstream and downstream primers and the Q0. (Table 2).

The bands from the 2° RESDA PCR reactions that got fragments were then cut out and purified using Qiaex gel purification protocol from Qiagen Company and sent for Sanger sequencing at Genewiz Company (Cambridge, Ma).

BLAST searches were done using the sequences of each mutant strain from the Sanger sequencing to identify the locations of the mutated genes in the *C. reinhardtii* v5.5 genome sequence in the Phytozome database v12.1 (Goodstein, et al. 2012). A sample sequence and the genome are shown below in FIG 5 (in this, mutant strain *3B3* was used). The E values (Expect values) from the genome searches gave us information on the significance of the hits; percent identity and percent similarity also gave us information on how the DNA sequences that were used in the BLAST searches were similar to and different from *C. reinhardtii* genome (FIG 5).

A.

3B3_Pst_UP_UPS
>Sample DNA trace | 843 bp
NNNNNNNNNNNNNNNCGNNNNNTAGCGGAGTGCGATCACAAGCTCGAGTGGCCTGTGT
AGAAGTGGTAGTGATCTAGGTGTTTGAATATGGCTTTGGTAGCTCGCTATAATGTCTTTGCAAT
CGGGGGCCTGGCTATTTAAACAGCGCTCGCCCTGAGCGGCATCGGAGCGCCCATGCAGCC
CCGAAGGAGCTTCGGGGGGTCGAAGCATCATCGGTGTTGCATGCAGCGCCGGGAAGCCGT
CTCGCAGCCCGCCCTACCTTTTGCTGGAAGTGTCATAGCGCAAGAAAGAAGCTTGAGACAGT
GACAGAGTACACGGTCGCCCGATTCGTGGCCCGATCAGCCAGGGAGCCCAGCGCCCGCCA
ACATGGCGCGAAAGGGCCCTACTGTTGCACGTCGGAAGCGGTATGTCCATCAGTCCATGTG
CTCCATGCTCTTTACATGCTTTGATGCATATATGCACGCGGCATGCTGTTACTTATGCCATAGT
TCAGTGTGTGTACAGGGACACCGTGGGACACGCCATGGGTTGGCCGCTCATCCGGTCATCC
CCAGCACCCCTCCCTACGATAATCATAGCGCGGATGGCAGCTAATGTACACCCACCGGCCAC
TTGGGGCGCTATCGTTCATCGGTCATGCATCTCGCCAAACATGTCANCTCATGCACCACCCG
TATGCCCTCTTTGTCGGTCCGTCTACCCCATGTCGNACCTTCNCTGTGCCATGCTTTCCTGN
GCTTTAAAAACNGGCANCCGCTACCATATACAAAGTACCAACACAGCCANGTGCGCGTCCTC
NNCNNNNANTNGAACNNNNNCNTTNNNNGCCNAANTNNNNNNNC

B.

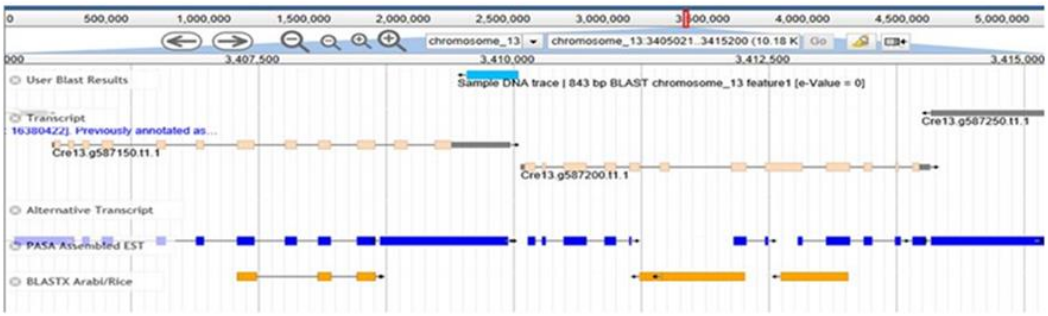

FIG 5. (A). A sequence of the delay mutant, *3B3* from the Sanger sequencing. This sequence was used to locate the mutated gene in the *C. reinhardtii* genome, and the chromosome on which the gene was mutated. This search was done for all the mutant strains by using NCBI BLAST and/or Phytozome. **(B).** Phytozome genome browser view of the3B3 insertion site identified by the RESDA PCR product sequence labeled with sample DNA. The BLAST searches in Phytozome also gave visual representations of the genome, the chromosomes on which the mutated gene were located, and the names and notations of the mutated genes. Also, other information such as the E values and whether the mutated genes were known or not were given by the BLAST searches as well.

- FURTHER IDENTIFICATION OF THE PHENOTYPES OF THE

SELECTED MUTANTS:

A regeneration curve was done to visually represent the duration in regenerating flagella after deflagellation for both the wild-type strain and the mutants. The deflagellation for this experiment was done manually in a 50ml beaker. 30ml of mutant cells was put in the 50ml beaker and put on a stir plate at medium speed. 6.7ml of 0.03M acetic acid was

pipetted into the beaker using a 10ml pipette to drop the pH. A sample of the cells were put on a petri dish and screen under a microscope for loss of flagella. After 2 minutes, 6.7ml of 0.03M KOH was pipetted into the beaker containing the acid and the cells to bring the pH back to 7. Samples of the strains were taken at different time points for imaging (Axio Observer – AxioCam MRm) during the regeneration process after their flagella were excised. 500µl of the cells were fixed in glutaraldehyde by adding an equal volume of 2.5% glutaraldehyde to give a final concentration of 1.25% glutaraldehyde before imaging (40X / 0.62ph2 LD plan – NEOFLAUR). Using phase contrast, 30 measurable flagella were imaged (regeneration curve). Images of cells from the deflagellation experiment were analyzed and the flagella were measured using FIJI.

For luciferase assays, 500µl of the cells was pipetted from the 50ml beaker after the base (0.03M KOH) was added at each time point into a well-labelled 1.5ml test tubes. The tubes were then snap frozen in liquid nitrogen. The test tubes containing the cells were then stored in a -80° freezer. 50µl of 0.01µM Coelenterazine, a luciferin substrate (Gold Biotechnology, St. Louis, MO), was used as the substrate for the luciferase assay experiment. Each well contained 100µl of the cells and 100µl of TAP Medium (Gorman and Levine, 1965). The luciferase assay equipment (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski,VT) was primed with ethanol and water, and purged with air before it was used to inject the 50 microliters of 0.01 M substrate.

RESULTS:

- ISOLATION OF MUTANTS:

C. reinhardtii are known for the ability to regenerate their flagella within 60 – 90 minutes after pH-shock-stimulated deflagellation. During regeneration, *C. reinhardtii* cells increase the expression of hundreds of genes encoding flagellar proteins. The mechanism behind this increased expression is poorly understood. However, previous experiments indicated that cells with inabilities to upregulate a reporter of gene induction during regeneration also had delay in regeneration of flagella (Brown and Witman, in preparation). Therefore, the purpose of this experiment was to isolate mutants that had delay in regenerating their flagella after insertional mutagenesis and the stimulation of the deflagellation process by acid-shocking the cells. Mutants that showed delay in regeneration were selected for characterization of their phenotypes by doing additional experiments (FIG 1).

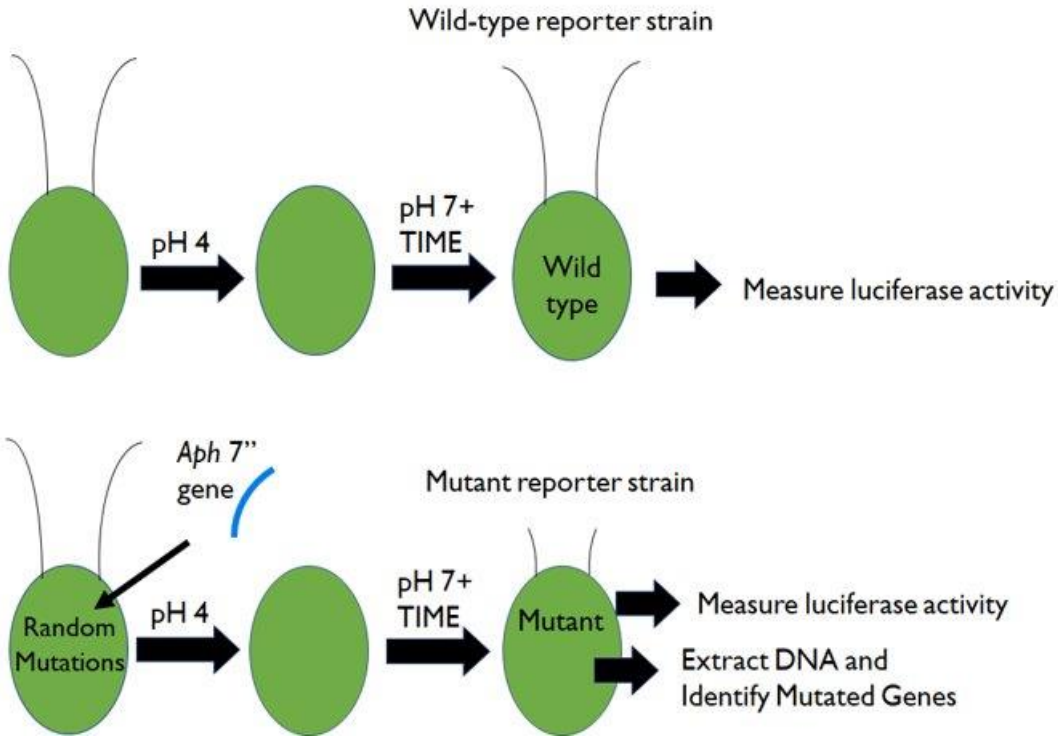

FIG 1: The overview of the experiments that were done. Wild-type *Chlamydomonas reinhardtii* can regenerate flagella to normal length with time and at pH7 after an induced deflagellation at pH 4. The wild-type *C. reinhardtii* contains an LC8 reporter construct that was designed to measure gene expression or the upregulation of luciferase during cell growth and after deflagellation. However, the random insertion of the

aph 7" gene creates *C. reinhardtii* mutants some of which are not able to regenerate flagella over the same time period as the wild-type cells. The mutated genes were identified by extracting the DNA of the mutant strains. Also, the mutants were screened for the ability to upregulate luciferase controlled by the dynein light chain *LC8* gene promoter.

C. reinhardtii mutants were generated by random insertions of a*ph7"* selectable marker into the cells' genomes (Berthold, et al, 2002). This resulted in the generation of 3000 hygromycin-resistant *C. reinhardtii* colonies including 42 flagella mutants (Table 1). 14 of these 42 mutants exhibited delays in flagellar regeneration. The delay was exhibited by the mutants slowly regrowing their flagella after deflagellation. Within 60-90 minutes, the wild-type *C. reinhardtii* used as a control had regenerated their flagella and assumed normal movement. The mutants on the other hand took a longer time to regenerate their flagella to the full length. The other 28 isolated mutants had flagellar defects such as palmelloid where the cells failed to separate from the mother cell wall after cell division (Li, et al. 2018), slow and shaky swimming, etc. (table 1).

For the purposes of multiple experiments, the workload was distributed in order for all the isolated mutants to be analyzed by subdividing the 42 mutants among other members of the Brown research group. The experiments below were only focused on 14 mutants; 3 of the mutants (3B3, 5D9, and 2A8) showed potentials to be very interesting (Table 1).

- RESDA PCR AMPLIFIED MULTIPLE SPECIFIC BANDS:

To further define the mutated genes responsible for the delay in flagellar regeneration, the genomic DNA was extracted from each *C. reinhardtii* mutant strain and amplified with the RESDA PCR protocol using PCR primers specific to the hygromycin cassette and different degenerate primers (Gonzalez-Ballester, et al. 2005) (FIG 2).

RESDA PCR was used to amplify the specific bands from both the primary and secondary PCR that was done.

As part of the selection process of the mutants to work with or to send for sequencing, all the primary and secondary PCR reactions were analyzed on a 1% agarose gel. On the gel, there were 8 reactions per mutant strain with each strain being as a result of the mixture of both the degenerate primers and the upstream and downstream primers. The purpose of running the primary reaction on a gel before doing the secondary PCR reaction was to check if there were any bands generated by the primary reactions. The bands for the secondary reactions were more specific than the bands from the primary reactions when compared with each other. This was noticeable by the appearance of the agarose gels used to run the reactions. The bands from the primary reaction were smeary compared to the bands from the secondary reactions which were tighter and more prominent. (FIG 2).

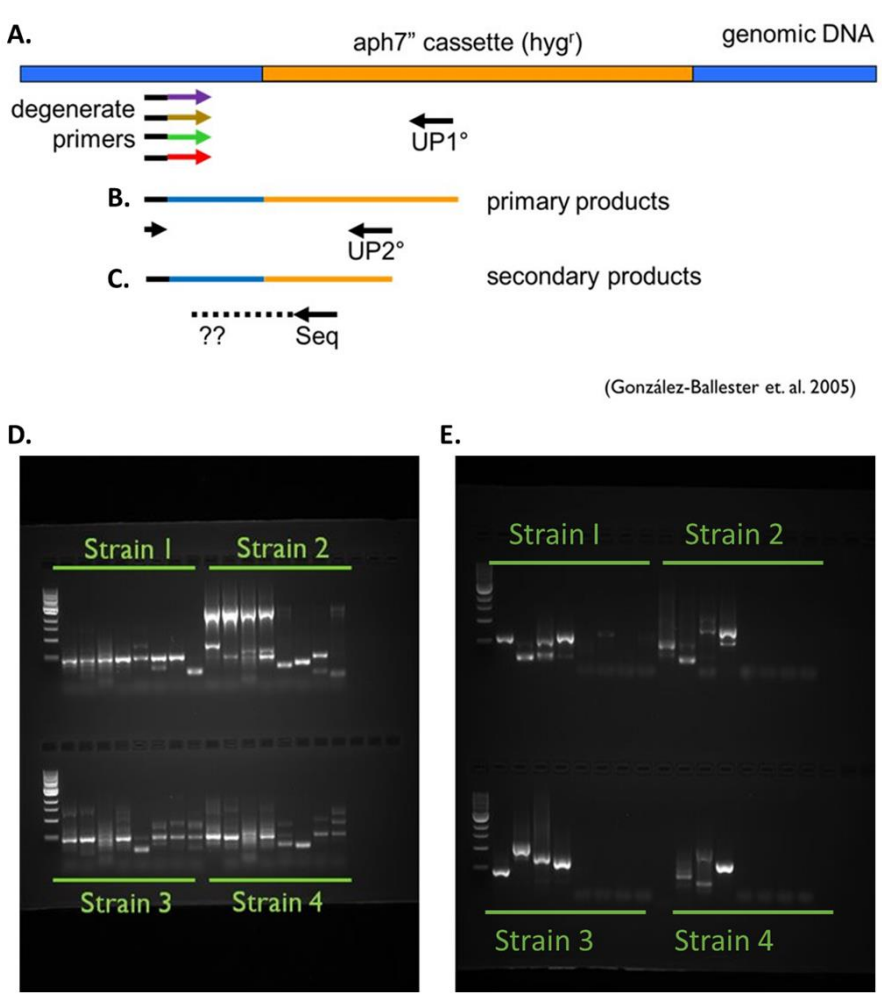

FIG 2: RESDA PCR reaction was done to amplify the genomic DNA of *C. reinhardtii* after the *Aph 7"* (orange) gene was inserted into the genomic DNA (blue). **(A).** Four degenerate primers *Alu*I (purple), *Pst*I (gold), *Sac*II (green), and *Taq*I (red) with a common QO sequence (black) were used with the upstream primer (UP1, yellow) to run the primary PCR reactions. **(B).** Any of the four primers have flexibility in where they bind in the genome. The 2° reaction is a nested reaction using the specific QO sequence (black) plus upstream secondary primer (UP2°). Additional specificity was achieved by using nested reaction. Only the upstream situation is shown here, but the same occurs with downstream primers as well. **(C).** 2° PCR reactions were done to further amplify the genomic DNA and to locate the inserted gene in the cell's genome and the gene mutated. The 2° reaction products with bands were Sanger sequenced. **(D).** The primary reactions were analyzed on an agarose gel before they were used as templates for the 2° reactions. Each mutant strain had all the four degenerate primers with the upstream primer and the four degenerate primers with the downstream primer. Each strain had 8 primary reactions. **(E).** The 2° reactions were also analyzed on gel and the reaction with the tight and specific band were cut, purified, and Sanger sequenced for the DNA sequences. D and E are from 2 representative RESDA PCR experiments.

Secondary PCR was done for all the 14 selected mutants, then a few of the mutants were selected for further experiments after analyzing whether or not they got bands from the secondary reaction. 9 mutants generated secondary reactions bands. Meaning

that, 9 mutant strains with different combinations of the upstream and downstream primers together with the degenerate primers had bands at the end of the secondary PCR reaction. Bands generated were almost evenly distributed among the upstream and downstream primers with 12 of the bands from the upstream end, and 13 of the bands on the downstream end. A total number of 25 bands were generated from the 9 mutant strains using the four degenerate primers. Of all the four degenerate primers, *Alu*I generated 8 bands; *Pst*I generated 5 bands; *Sac*II generated 5 bands; and *Taq*I generated 7 bands. Of the 25 bands mentioned, 14 of them were successfully sequenced (table 2).

- MUTANT STRAINS WERE SEQUENCED AFTER PRIMARY AND SECONDARY PCR REACTIONS:

The bands from the 2° PCR reactions that were more specific were cut out and purified. The purified bands which were sent for Sanger sequencing using an upstream-specific or downstream-specific sequencing primer revealed the DNA sequences (sequences of A, T, G, and C DNA nucleotides) specific to the DNA amplified from the mutant strain. The sequences helped us to determine the genes that were mutated in the cells' genomes. All 25 bands generated from the 8 mutants were Sanger sequenced, but 14 bands were successfully sequenced (table 2).

- THE SEQUENCED RESULTS WERE USED TO SEARCH THE *C. reinhardtii* GENOME:

A database called Phytozome (Goodstein, et al. 2012) was used to do a BLAST search to identify the mutated genes in the cells' genomes. NCBI BLAST (Basic Local Alignment Search Tool - Several variants of BLAST compare all combinations of nucleotide or protein queries with nucleotide or protein databases with short matches be-

tween two sequences) (Johnson, et al. 2008.) searches were done for any sequences that did not have a clear match to the *C. reinhardtii* genome. This is because their sequences resulted in no matches to any gene in the *C. reinhardtii* genome when searches were done in Phytozome (Goodstein, et al. 2012). The searches gave us information about the chromosomal location and about how the gene is currently annotated in the *C. reinhardtii* version 5.5 genome sequence. Literature searches with the BLAST results revealed information about any of the mutated genes in *C. reinhardtii* cells' genomes that had already been identified by other cell biologists.

Each of the mutants had different BLAST results because of the different chromosomal insertion sites of the *aph7"* gene. The results for all the 8 mutant strains were put into 4 categories into long matches to *C. reindhartii*; short matches to *C. reinhardtii*; matches to the hygromycin cassette; and matches to NCBI sequences (Table 2). The promoter for the hygromycin cassette was the B tubulin promoter on chromosome 12 and the terminator for the cassette is from the Rubisco small subunit gene on chromosome 2. Therefore, matches to either of these genes (Beta-Tubulin and RuBisCo) are mostly likely just matches to the hygromycin cassette:

- **3B3**

Delay mutant 3B3 generated one band, *3B3 PstI UP* (a combination of the 3B3 strain, *Pst*I degenerate primer and the upstream primer) which was Sanger sequenced. The sequence had 2 hits in the *C. reinhardtii* genome when a BLAST search of the *C. reinhardtii* genome v5.5 was done (Goodstein. et al. 2012). One of the 2 hits had a long match to the cell's genome and the other had a match to the hygromycin cassette. The long match was to chromosome 13 with 98% identity (495/505) and an E value of 0.0.

The mutated gene was identified as Cre13.g587200 which is annotated as Phosphatidyl-inositol glycan, class W (PIG W) on the 3' end. The other hit was a match to the inserted hygromycin cassette on chromosome 12. The match had 100% identity (269/269) to the Beta - Tubulin 2 on chromosome 12. The E value for the match was 3.4E-136.

- **2A8**

Mutant strain 2A8 which had palmelloid phenotype had 2 bands generated; *2A8 AluI UP* (a combination of the 2A8 strain, *Alu*I degenerate primer, and the upstream primer) and *2A8 TaqI UP* (a combination of the 2A8 strain, *Taq*I degenrate primer, and the downstream primer). Each of the 2 bands had different amounts of hits in the cell's genome.

Band *2A8 TaqI* UP had one long hit to the hygromycin cassette. The hit was a match on chromosome 12. The match had 96.2% (182/186) identity and an E value of 2.6E-85. The mutated gene was identified as Cre12.g549550 and annotated as Beta - Tubulin 2.

Band *2A8 AluI UP* had 4 hits when a search was done in Phytozome (Goodstein, et al. 2012). The first hit was a long match to the cell on chromosome 11. The identity of the match was 88.1% (111/126) with an E value of 3.2E-39. The gene was identified as Cre11.g476050 and annotated as Flagella outer dynein arm heavy chain gamma. The hit was a long match to the hygromycin cassette on chromosome 12 with 100% identity (272/272). The match had an E value of 4.9E-138 and the mutated gene identified was Cre12.g549550 and annotated as Beta - Tubulin. The third hit was a short match to the hygromycin cassette on chromosome 2. The identity of the match was 100% (78/78) with an E value of 1E-32. The mutated gene was identified as Cre02.g120150 and annotated as RuBisCo (Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2). The

fourth hit was a short match to the cell's genome on chromosome 14. The identity of the match was 85.7% (36/42) with an E value of 5E-5. The mutated gene was identified as Cre14.g624950 and annotated as Flagella inner arm dynein 1 heavy chain beta.

- **5D9**

Delay mutant 5D9 had one band generated; *5D9 SacII DP*. The band was Sanger sequenced and the sequence was used to do a BLAST search in Phytozome (Goodstein, et al. 2012). Band *5D9 SacII DP* had one hit to the cell's genome. The hit was a long match on chromosome 1. The identity of the match was 83.1% (157/189) with an E value of 1.6E-50. The identified mutated gene was Cre01.g043050 annotated as TVP38/TMEM64 FAMILY MEMBRANE PROTEIN YDJX.

- **1B10**

Delay mutant 1B10 had 3 bands generated: *1B10 AluI UP* (4 hits): a short match to a gene on chromosomes 6 with identity of 86% (37/43) and an E value of 2.6E-2. The mutated was identified as Cre06.g260500 and annotated as non-specific serine/threonine protein kinase / Threonine-specific protein kinase; a short match to a gene on chromosome 17 with identity of 95.8% (23/24) and an E value of 9.1E-2. The mutated was identified as Cre17.g698800 and annotated K06664 - peroxin-2 (PEX2, PXMP3); a short match to a gene on chromosome 13 with identity of 92.3% (24/26) and an E value of 9.1E-2. The mutated gene was identified as Cre13.g584750 and annotated K18162 - NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 5; and a short match to a gene on chromosome 12 with identity of 99% (395/396) and an E value of 0.0. The mutated was identified as Cre12.g549550 and Beta – Tubulin 2. *1B10 TaqI UP* (one hit): a long match to the hygromycin cassette on chromosome 12 with identity of 100%

(273/273) and an E value of 1.6E-138. The identified mutated gene was Cre12.g549550 and annotated as Beta – Tubulin 2. And *1B10 PstI DP* (3 hits): a long match to the hygromycin cassette on chromosome 12 with identity of 98.7% (303/307) and an E value of 1.3E-151. The identified mutated gene was Cre12.g549550 and annotated as Beta – Tubulin 2; a long match to the hygromycin cassette on chromosome 2 with identity of 90.9% (159/175) and an E value of 1.2E-63. The identified mutated gene was Cre02.g120150 and annotated as RuBisCo (Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2); and a short match to the hygromycin cassette on chromosome 13 with identity of 83.3% (30/36) and an E value of 9.1E-2. The identified mutated gene was Cre13.g591300 and annotated as PTHR19862:SF14 - WD REPEAT-CONTAINING PROTEIN 48.

- **7G5**

7G5, a mutant with its phenotype characterized as periodic spinning had 2 bands from the reaction. The bands generated were *7G5 PstI UP* and *7G5 TaqI DP*. Sanger sequencing gave the sequences of the 2 bands that were later used to search the cell's genome for any matches. Band *7G5 PstI UP* had one hit when the sequence was used in Phytozome (Goodstein, et al. 2012). The hit was a long match to the hygromycin cassette in the cell's genome on the chromosome 12. The identity of the match was 99% (198/200) with an E value of 3.1E-97. The mutated gene was identified as Cre12.g549550 and annotated as Beta – Tubulin 2. Band *7G5 TaqI DP* also had one hit with a long match to a gene on chromosome 2. The identity of the match was 80.8% (122/151) with an E value of 1.5E-37. The mutated gene was identified as

Cre02.g120150 and annotated as RuBisCo (Ribulose-1, 5-bisphosphate carbox-ylase/oxygenase small subunit 2).

There were 4 mutant strains with bands that gave hits to species other than *C. reinhardtii*. The strains included 6D3 palmelloid mutant strain (2 bands from the secondary reaction, *6D3 PstI DP* and *6D3 SacII DP*), 7D7 single non-motile mutant strain (6 bands from the secondary reaction: *7D7 AluI UP, 7D7 TaqI UP, 7D7 AluI DP, 7D7 PstI DP, 7D7 SacII DP, and 7D7 TaqI DP), 6D1* delay mutant strain (4 bands from the secondary reaction: *6D1 AluI UP, 6D1 AluI DP, 6D1 PstI DP, and 6D1 SacII DP),* and 5D5 delay mutant strain (3 bands from the secondary reactions: *5D5 SacII UP, 5D5 TaqI UP,* and *5D5 TaqI DP*).

Out of the 9 mutant strains that were Sanger sequenced, the 3 mutants (*3B3, 5D9,* and *2A8*) that showed potentials to be interesting were selected for further experiments and further characterization their phenotypes. Most characterizations were done for the two delay mutant strains.

- THE WILD-TYPE STRAIN HAD LONGER AVERAGE LENGTH THAN THE *5D9* MUTANT STRAIN BEFORE AND AFTER DEFLAGELLATION:

The lengths of the flagella for both the wild-type and the delay mutant strain *5D9* were measured prior and during the deflagellation experiment. It was observed that the vegetative cells of both strains had different flagella lengths (FIG 3). The wild-type strain had an average longer flagella length than the mutant strain. The average lengths were measured from 30 cells from each strain prior to deflagellation experiment. The result showed the 30 wild-type cells used for the measurement to have an average length of 12.40013 microns whereas the 30 mutant cells were shown to have an average length of 7.388592

micron. This could have resulted from the insertion of the *aph7"* gene. The possible explanation could be that the mutated gene disrupts the proteins needed for flagella growth or the mechanisms of flagella growth got slowed down by the mutation. In construction of the figures, 30 cells from each strain at each time point were used. However, because 7.4% of cells of the total number of the *5D9* cells were flagellated at time point 0, we took 7.4% of 30 total number of cells for the flagella length measurement. Similarly, 12% of the *5D9* cells were flagellated at time 15 and same steps were taken in measuring the length of the flagella at that time point.

- THE MUTANTS WERE SLIGHTLY ABLE TO UPREGULATE THE LUCIFERASE:

To build the *LC8* construct, a promoter from the *LC8* gene was connected to the coding region that codes for protein from the *Gaussia* princeps luciferase. The *GLuc* gene encodes the *Gaussia* luciferase protein that generates a burst of blue light when exposed to coelenterazine (Yu, et al. 2018). The construct expresses the GLuc protein under the control of the *LC8* promoter (Brown & Witman, unpublished).

A luciferase assay experiment was done to show whether the upregulation of the *LC8* reporter construct in the cells were affected by the inability of the mutants to regenerate their flagella as fast as the wild-type strain. For this experiment, the wild- type *C. reinhardtii* had the *LC8* construct that was designed to monitor the expressing of the *LC8* gene which encodes a flagella dynein light chain. It was observed that the insertion of *aph7"* disrupted the mutants' ability to upregulate the *LC8* luciferase reporter. The mutants were slightly able to upregulate the luciferase. The initial upregulation of luciferase by the *5D9* mutant strain was about the same as the control wild-type D10. However after

time 30mins, the rate of upregulation by the wild-type strain was almost as twice as the mutant strain (FIG 3). This is because the positive – control wild-types cells expressed the luciferase construct after deflagellation over the same period of time as the mutant strains were given. The experiment was interpreted using a fold expression graph (FIG 3). The *3B3* mutant strain also showed a slight ability to upregulate luciferase. The up-regulation was lower than that of the *5D9* when compared each other (results not shown).

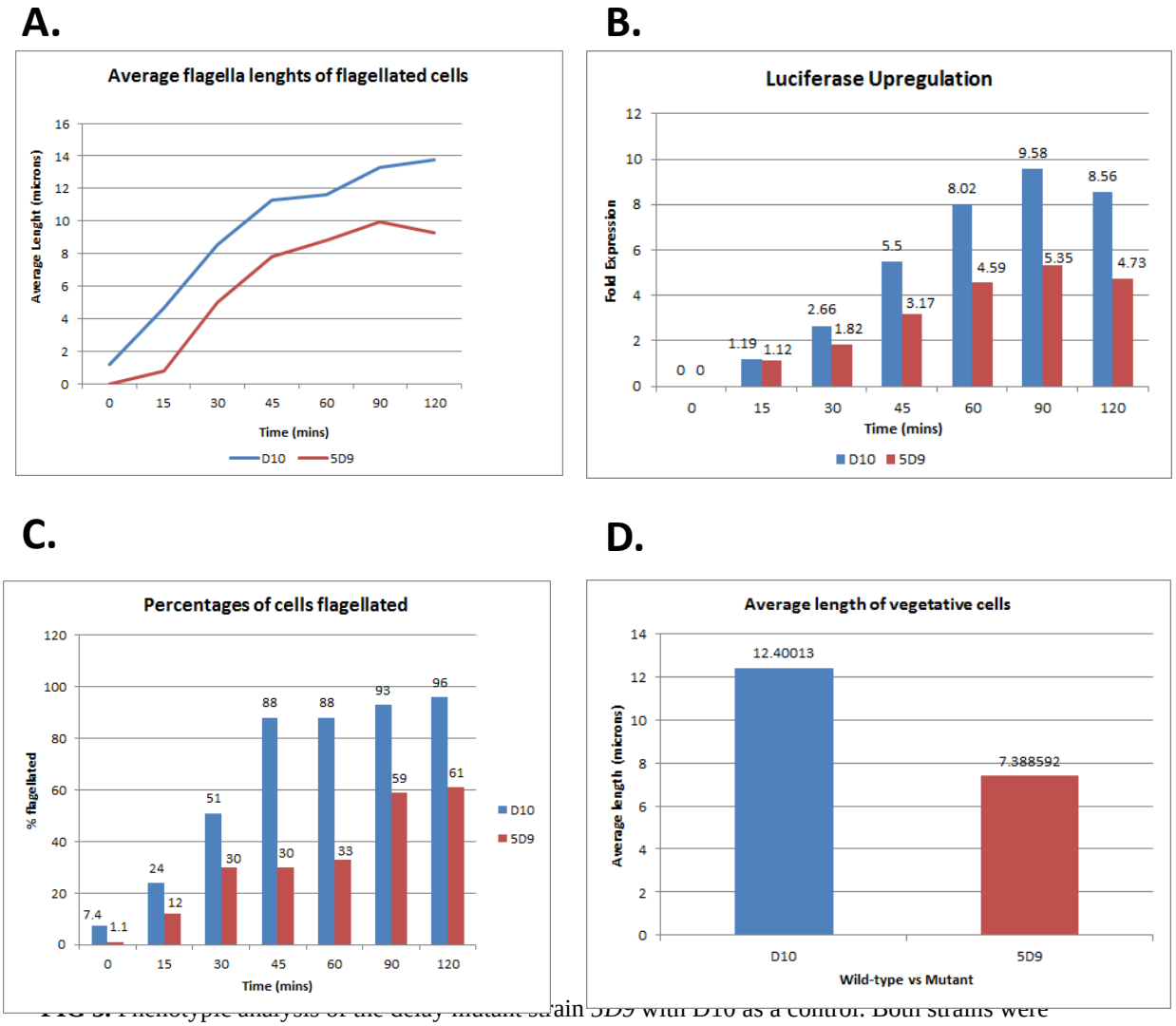

FIG 3. Phenotypic analysis of the delay mutant strain *5D9* with D10 as a control. Both strains were deflagellated by pH shock (Branch et al. 2013) and screened for delay in flagellar regeneration and luciferase upregulation. **(A).** The *5D9* mutant strain showed a slight delay in flagellar regeneration compared with the D10 wild-type strain. ***(B).*** The upregulation of luciferase by *5D9* was lower than the upregulation of luciferase by D10 during regeneration of flagella. However, the two strains relatively had the same initial upregulation at 15 minutes, the mutant strain eventually started to show delay in upregulation of luciferase. **(C).** Percentages of cells flagellated at each time during flagellar regeneration. **(D).** A comparison of the

vegetative cells of the wild-type strain, D10 and the mutant strain, 5D9 to depict the difference in the average flagella lengths prior to deflagellation. All the figures were constructed with at most 30 measurable flagella of each strain at each time point in each experiment.

DISCUSSION:

The purpose of this experiment was to generate mutants that had delay in regenerating their flagella. *Aph7"* gene was used because of it confers resistance to hygromycin B (Borovinskaya, M. 2008). *C. reinhardtii* cells are sensitive to aminoglycoside antibiotics such as Hygromycin B. However, the *aph7"* gene (bacterial genes encoding aminoglycoside-inactivating phosphotransferases) from *Streptomyces hygroscopicus* has a similar codon to *C. reindhardtii* which allows it to confer resistance to hygromycin B (Berthold, et al. 2002).

3000 hygromycin-resistant mutants were generated by using this transformation technique. 14 of the mutants exhibited delay in regenerating their flagella and 28 had phenotypes, such as being palmelloid, failing to swim, or having abnormal swimming behavior, indicating likely flagella defects. To identify the mutated genes causing the phenotype, RESDA PCR was done for the mutants that showed potentials to be interesting. The DNA samples were Sanger sequenced and BLAST searches were done to identify the insertion sites of the hygromycin cassette which contained the *aph7"* resistant gene. Although Phytozome (Goodstein, et al. 2012) was primarily used for the BLAST searches, there were some mutant strains that did not have any hits in Phytozome (Goodstein, et al. 2012). Therefore, NCBI BLAST searches had to be done for such strains (Johnson, et al. 2008) (Table 2). Phytozome provides a view of the evolutionary history of plant genes and algae gene families and individual genes (Goodstein, et al. 2012) whereas NCBI BLAST compares all combinations of nucleotide or protein queries with

nucleotide or protein databases (Johnson, et al. 2008). Therefore, Phytozome searches were limited to the genome of *C. reinhardtii* and NCBI BLAST searches were opened to all homologs of the annotated gene and multiple genomes of multiple species.

After the searches, it was observed that some of the strains had long matches to the genomic DNA flanking the hygromycin cassette insertion site and others had short matches. This observation is explained in Li, et al. (2016) that the short matches could be due to multiple insertions in a single mutant strain, introduction of DNA from a distant locus on one or both sides of the cassette, and or multiple mutants growing or picked within the same colony. The second implication makes more sense to this experiment since some of the short matches were from different chromosome even within the same strain. For instance, mutant strain *1B10* had 4 short matches to its genome (Table 2). Other experiments that were done for 3B3 (results not shown) and 5D9 showed that the 5D9 had a slight delay in regenerating flagella. Also, the mutant strain an initial delay in upregulating luciferase but the rate of upregulation was the same in both the mutant and the wild-type strain (FIG. 5).

The three most interesting mutants that further experiments primarily focused on were delay mutants *3B3* and *5D9,* and *2A8* with flagellar defect. This was due to their interesting genes.

The mutated gene for the 3B3 band *3B3 PstI UP,* was Cre13.g587200 which is annotated in the *C. reinhardtii* genome v. 5.5 available in Phytozome (Goodstein, et al. 2012) Phosphatidylinositol glycan, class W (PIG W). This gene encodes an inositol acyltransferase that acylates the inositol ring of phosphatidylinositol in an early step of Glycosylphosphatidylinositol (GPI) biosynthesis (Chiyonobu, et al. 2014). GPI is an im-

portant glycolipid protein that anchors several kinds of proteins to the cell surfaces in humans. It is not known yet if this protein performs the functions in other organisms. Because of its importance, a deficiency in PIGW which will prevent the acylation of the GPI protein can cause disease such as West syndromes stated in Chiyonobu, et al. (2014). Proteins containing a GPI anchor are functionally diverse and play important roles in signal transduction, prion disease pathogenesis, immune response, and the pathobiology of trypanosomal parasites (Chesebro, et al. 2005). This could be one the explanations of the reasons why the 3B3 mutant had a delay in regenerating flagella. Since GPI protein also plays a role in signal transduction, it makes sense for a mutation to disrupt this pathway. This could mean that the insertion of the *aph7"* gene inhibited the mutated the PIGW gene which inhibited the acylation of GPI. This could have resulted in the inability of the 3B3 mutant cells to signal the nucleus for flagellar regeneration after the induced deflagellation. In Chiyonobu, et al. (2014) a patient with GPI deficiency showed severe developmental delay with dysmorphic facial features and hyperphosphatasia. These are characteristics often seen in inherited Glycosylphosphatidylinositol deficiency. The patient had decreased surface expression of GPI-anchor proteins which was identified to be mutations in PIGW. Hence PIGW is an important gene that should be taken into consideration when screening for age-dependent epileptic encephalopathy West syndrome as well as a syndrome exhibiting hyperphosphatasia and cognitive disability (HPMRS5) in humans (Chiyonobu, et al. 2014). Also, the connection between the diseases caused by the deficiency in PIGW and the mutation in *C. reinhartdii* shows how important *C. reinhardtii* is for studying ciliopathies in humans. Also, the connection between the diseases caused by the deficiency in PIGW and the mutation in *C. reinhartdii* shows how im-

portant *C. reinhardtii* is for studying genetic disorders such the autosomal recessive disorder, HPMRS5 in humans (Chiyonobu, et al. 2014).

The *5D9 SacII DP* band from the 5D9 mutant strain, also a delay mutant, had the mutated gene identified as Cre01.g043050 annotated as TVP38/TMEM64 FAMILY MEMBRANE PROTEIN YDJX. The protein encoded by this gene has homology to 2 different proteins families: TVP38 and Transmembrane protein 64 (Tmem64). Tmem64 has seven predicted transmembrane domains and a conserved SNARE domain. It regulates calcium during osteoclast differentiation. A study by Kim, et al. (2013) showed decrease in osteoclast differentiation and increase in bone mass when Tmem64 was knocked down in mice. It was discovered that Tmem64 interacts with sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) and modulates its activity. Impairment of Ca^{2+} signaling pathways in the absence of Tmem64 reduces SERCA2 activity resulting in inefficient osteoclast differentiation and function. Also, deficiency in Tmem64 reduces CREB activity. CREB (cyclic AMP response element-binding protein CREB) is activated by phosphorylation in response to several signaling pathways, including cAMP, calcium, stress and mitogenic stimuli. Phosphorylation of CREB at Ser133 in response to cAMP stimulus is enough to induce target gene expression, but additional promoter-bound transcription factors are required for induction in response to non-cAMP signals (Mayr and Montminy, 2001).

TVP38 on the other hand together with other TV proteins are integral membrane proteins and colocalize with T-SNARE affecting a late Golgi compartment (Tlg2) and early endosome compartments. These compartments are involved in vesicular traffic (Inadome, et al. 2007). They are vesicle-associated proteins involved in the transporting of

materials in the Golgi apparatus that are conserved in chloroplasts and other prokaryotes. Homologs of Tvp38 are not only conserved in prokaryotes, but also in higher eukaryotes, including humans. A study by Inadome, et al. (2007) suggested that Tvp38 plays a role in vesicular trafficking along the secretory pathway and might be involved in organizing vesicular structures.

It is rational to suggest that the mutated gene annotated as TVP38/TMEM64 FAMILY MEMBRANE PROTEIN YDJX functions to transport signaling molecules or transcription factors that are involved in signaling the nuclei to regenerate flagella in *C. reinhardtii*. Therefore if there is a mutation that slows this gene down or disrupts the signaling pathway, it could lead to delay in regeneration flagella. This is evident in this experiment; delay in flagellar regeneration as a result of mutated TVP38/TMEM64 FAMILY MEMBRANE PROTEIN YDJX.

Less is known about the role of TVP38 in vesicular trafficking in and according to Keller and Schneider (2013), a series of proteins, including Tvp38-homolog, might play a role in initiation, assembly, budding/tethering of vesicles and/or membrane fusion. Flagella proteins are transported to the base of the flagella by vesicular trafficking and budding. Other membrane proteins such as IFT20 protein are recruited into the flagella vesicles as adaptors in addition to the direct binding of the vesicle membrane proteins (Emmer, et al. 2010).

This shows the importance of vesicular transport/budding in flagellar regeneration that the role of TVP38/TMEM64 FAMILY MEMBRANE PROTEIN YDJX in this mechanism could help us understand it better.

The 2A8 which had a flagella defect with a palmelloid phenotype had 2 bands with one of the bands (*2A8 Alu*1 *UP)* having 4 hits to different flagella genes. 2 of the 4 genes were long hits to the hygromycin cassette and were identified as Beta – Tubulin 2 on chromosome 12 and Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2 on chromosome 2. The other 2 were to already known flagella genes and were identified as Flagella outer dynein arm heavy chain gamma on chromosome 11 and Flagella inner arm dynein 1 heavy chain beta on chromosome 14. Dyneins are very important in translocation. They are the large molecular motor that translocate to the (-) ends of microtubules. Organisms with cilia or flagella express approximately 14 different dynein heavy chain genes, each gene encodes a distinct dynein protein isoform, and each isoform appears to be functionally specialized (Asai and Wilkies, 2004). Dyneins are also involved in motile events occurring within cellular compartments other than the flagellum (King and Witman, 1988). The outer arm dynein from *C. reinhardtii* contains three subunits (alpha, beta, and gamma). An experiment by King and Witman (1988) suggested that the beta and gamma chains are structurally and possibly functionally related based on the same cleavage sites using endoproteases. This finding makes in the above mentioned identified genes interesting since the phenotype of the mutant strain is palmelloid. The identified mutated genes could potentially be explanations to the phenotype of this strain as well as to the finding by Li, et al. (2018) that the palmelloid phenotype in their experiment was to the failed release of daughter cells after cell division which demonstrates that the cells were aflagellated or had very short flagella. The phenotype of the mutant in their experiment was resolved by autolyzing the cells. Could this suggest that the under-

lying mechanism of the palmelloid is due to the failure of the mutated genes to assist in motile events that occur in the cells as describe by King and Witman (1988)?

Since these experiments were preliminary experiments, other experiments such as backcross and rescuing the mutant could be done in the future to confirm that the phenotypes of all the 3 interesting mutants were indeed caused by the mutated genes. Further phenotypic analysis would help us to better understand the mechanisms involved in flagellar regeneration and diseases caused by flagellar defects.

Table 1: Generated mutant strains with their phenotypes:

MUTANT NAME:	**PHENOTYPE**:
3B3	DELAY
5D9	DELAY
5D5	DELAY
1B10	DELAY
2A9	DELAY
2E10	DELAY
6D1	DELAY
8A9	DELAY
11F8	DELAY
12A10	DELAY
12C8	DELAY
16B7	DELAY
16C5	DELAY
21A2	DELAY
1E1	PALMELLOID
2A8	PALMELLOID
6D3	PALMELLOID
12B12	PALMELLOID
10H11	PALMELLOID
19D12	PALMELLOID
12H9	PALMELLOID
13B12	PALMELLOID
20E12	PALMELLOID
19G9	50% PALMELLOID; 50% SHAKY

	MOVEMENT
4C4	PERIODIC SPINNING
7G5	PERIODIC SPINNING
12B12	PERIODIC SPINNING
6E9	SLOW AND SHAKY MOVEMENT
12C6	SLOW AND SHAKY MOVEMENT
17A5	SLOW AND SHAKY MOVEMENT
15H6	SLOW AND SHAKY MOVEMENT
17A5	SLOW AND SHAKY MOVEMENT
6E11	SINGLE NON-MOTILE CELLS
7D7	SINGLE NON-MOTILE CELLS
7F11	SINGLE NON-MOTILE CELLS
11E8	SINGLE NON-MOTILE CELLS
18E4	SINGLE NON-MOTILE CELLS
*** CONTAMINATED STRAINS:**	
5C2	SINGLE NON-MOTILE CELLS
7D7	SINGLE NON-MOTILE CELLS
15G3	UNKNOWN PHENOTYPE
17A7	UNKNOWN PHENOTYPE
17A8	UNKNOWN PHENOTYPE
17A11	UNKNOWN PHENOTYPE
17C4	UNKNOWN PHENOTYPE

3000 colonies of cells were generated with random insertion of the *Aph 7"* gene. 42 mutants were generated with 14 expressing delay in regenerating flagella and 28 expressing flagella defects. 9 of the mutants with flagella defects had palmelloid phenotypes where the cells are not able to separate from the mother cell during the cell cycle due to the absence of flagella or the flagella not being able to secrete the enzyme needed for the normal breakdown of the mother cell wall; 5 mutants expressed slow and shaky movements compared to the Wild-type cells; 3 mutants expressed periodic spinning during movement; 7 mutant strains had phenotypes that were characterized as single non-motile cells because of the inability of the individual cells to swim/move with 2 having been contaminated; 1 mutant strain expressed mixed phenotypes with 50% palmelloid and the 50% with shaky movement; The phenotypes of 5 of the mutant strains were not determined due to contamination. The strains are categorized from most interesting to least interesting.

Table 2: Mutant strains with 2° RESDA bands were Sanger sequenced and BLAST searches were done:

STRAIN NAME	**LONG MATCHES TO *C. reinhardtii***	**SHORT MATCHES TO *C. reinhardtii***	**MATCHES TO HYGROMYCIN CASSETTE**	**MATCHES TO NCBI SEQUENCE**
HYGROMYCIN CASSETTE + LONG MATCHES				
3B3 *Pst*1 UP 2 HITS IN PHYTOZOME	Chromosome 13; E value: 0.0; Identity 98% (495/505). K05283 - phosphatidylinositol glycan, class W (PIGW).		Chromosome 12; E value: 3.4E-136; Identity: 100% (269/269). Beta – Tubulin 2.	
2A8 *Alu*1 UP 4 HITS IN PHYTOZOME	Chromosome 11: E value: 3.1E-39; Identity 88.1% (111/126); Flagella outer dynein arm heavy chain gamma.	Chromosome 14: E value: 5E-5; Identity: 85.7% (36/42); Flagella inner arm dynein 1 heavy chain beta.	Chromosome 12; E value: 4.9E-138; Identity: 100% (272/272); Beta – Tubulin 2. Chromosome 2; E value: 1E-32; Identity: 100% (78/78); RuBisCo (Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2).	
2A8 *Taq*I UP 1 HIT IN PHYTOZOME			Chromosome 12; E value: 2.6E-85; Identity: 96.2% (182/186). Beta – Tubulin 2.	
5D9 *Sac*II DP 1 HIT IN PHYTOZOME	Chromosome 1; E value: 1.6E-50; Identity: 83.1% (157/189). PTHR12677:SF			

	5 - TVP38/TMEM 64 FAMILY MEMBRANE PROTEIN YDJX			
HYGROMYCIN CASSETTE + SHORT MATCHES				
1B10 *Alu*1 UP 4 HITS IN PHY-TOZOME		Chromosomes 6; E value 2.6E-2; Identity 86% (37/43). Non-specific serine/threonine protein kinase / Threonine-specific protein kinase Chromosome 17; E value: 9.1E-2; Identity: 95.8% (23/24). K06664 - peroxin-2 (PEX2, PXMP3) Chromosome 13; E value 9.1E-2; Identity: 92.3% (24/26). K18162 - NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 5.	Chromosome 12; E value 0.0; Identity: 99% (395/396); Beta – Tubulin 2.	
1B10 *Pst*1 DP 3 HITS IN PHY-TOZOME		Chromosome 13; E value; 9.1E-2; Identity; 83.3% (30/36). PTHR19862:SF14 - WD REPEAT-CONTAINING PROTEIN 48	Chromosome 12; E value: 1.3E-151; Identity: 98.7% (303/307); Beta – Tubulin. Chromosome 2: E value: 1.2E-63; Identity: 90.9% (159/175); RuBisCo (Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2).	
1B10 *Taq*I UP			Chromosome 12;	

1 HIT			E value: 1.6E-138; Identity 100% (273/273). Beta – Tubulin 2.	
HYGROMYCIN CASSETTE ONLY				
7G5 *Pst*1 UP 1 HIT IN PHY-TOZOME			Chromosome 12; E value 3.1E-97; Identity 99% (198/200). Beta – Tubulin 2.	
7G5 *Taq*I DP 1 HIT IN PHY-TOZOME			Chromosome 2; E value 1.5E-37; Identity: 80.8% (122/151). RuBisCo (Ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit 2).	
MATCHES TO OTHER SPECIES				
6D3 *Pst*1 DP 0 HITS IN PHY-TOZOME				NCBI BLAST: Boechera divaricarpa GSS, clone B53-01-F_D01; E value: 0.0; Identity: 88% (685/777).
6D3 *Sac*II DP 0 HITS IN PHY-TOZOME				NCBI BLAST: hypothetical protein [Escherichia coli]; E value: 1E-11. Identity: 58% (38/65).
7D7 *Alu*1 UP				NCBI BLAST: Ty-

0 HITS IN PHYTOZOME				rosine-protein kinase JAK3 [Equus asinus]; E value: 8E-14; Identity 35% (79/226).
7D7 *Pst*1 DP 0 HITS IN PHYTOZOME				NCBI BLAST: Homo sapiens Janus kinase 3 (JAK3), transcript variant X1, mRNA; E value: 5E-156 Identity: 84% (448/532).
7D7 *Taq*I UP 0 HITS IN PHYTOZOME				NCBI BLAST: Homo sapiens B-cell CLL/lymphoma 11A (zinc finger protein); E value: 0.0 Identity: 94% (435/463).

The mutant strains that had secondary RESDA bands were Sanger sequenced. Genomic searches were done with the sequences from the secondary reactions. The BLAST searches were first done in phytozome (Goodstein. et al., 2012) and if there were no hits, NCBI BLAST search was done (Johnson, et al. 2008.). The hits from the searches were: strains that gave long hits to *C. reinhardtii* and to the hygromycin cassette; strains that gave short hits to *C. reinhardtii* and to the hygromycin cassette; strains that gave hits to the hygromycin cassette only; and hits to other species indicating likely PCR contamination/artifacts. The search results provided mutated genes and their locations in *C. reinhardtii*'s genome, the annotation of the genes, the percent identity of the generated sequences to the sequences of the genes in the genome, and the Expect values (E values) of the hits that inform us about the quality of the hits.

REFERENCES CITED:

1. Albee, A.J., Kwan, A.L., Huawen Lin, H., Granas, D., Stormo, G.D., Dutcher, S.K. (2013). Identification of Cilia Genes That Affect Cell-Cycle Progression Using Whole-Genome Transcriptome Analysis in *Chlamydomonas reinhardtti*. G3: Genes, Genomes, Genetics. 3(6):979-991.
2. Asai, D.J., Wilkies, D.E. (2004). The Dynein Heavy Chain Family1. Journal of Eukaryotic Microbiology. 51: 23-29.
3. Berthold, P., Schmitt, R., Mages, W. (2002). An Engineered Streptomyces hygroscopicus *aph 7″* Gene Mediates Dominant Resistance against Hygromycin B in *Chlamydomonas reinhardtii.* Protist. 153(4). 2002: 401-412.
4. Borovinskaya, M.A., Shoji, S., Fredrick, K., Cate, J. H. (2008). Structural basis for hygromycin B inhibition of protein biosynthesis. RNA (New York, N.Y.). 14(8): 1590-9.
5. Brown, J.M., Cochran, D.A., Craige, B., Kubo, T., Witman, G.B. (2015). Assembly of IFT Trains at the Ciliary Base Depends on IFT74. Current Biology. 25(12):1583-1593.
6. Brown, J.M., DiPetrillo, C.G., Smith, E.F., Witman, G.B. (2012). A FAP46 mutant provides new insights into the function and assembly of the C1d complex of the ciliary central apparatus. J Cel Sci. jcs.107151.
7. Brown, J.M., Mosley, M., Montes-Berrueta, D., Hou, Y., Yang, F., Scarbrough, C., Witman, G.B., Wirschell, M. (2017). Characterization of a New oda3 Allele, oda3-6, Defective in Assembly of the Outer Dynein Arm-Docking Complex in *Chlamydomonas Reinhardtii*. Plos One.12 (3).
8. Chesebro, B., Race, R., Kercher, L. (2005). Scrapie pathogenesis in brain and retina: Effects of prion protein expression in neurons and astrocytes. Journal of NeuroVirology 11: 476.
9. Chiyonobu, T., Inoue, N., Morimoto, M., Kinoshita, T., Murakami, Y. (2014). Switching on Cilia: Transcriptional Networks Regulating Ciliogenesis. J Med Genet. 51(3):203-7.
10. Choksi, S.P., Lauter, G., Swoboda, P., Roy, S. (2014). Development. Oxford University Press for the Company of Biologists Limited.
11. Craige, B., Brown, J.M., Witman, G. B. (2013). Isolation of *Chlamydomonas* flagella. Current protocols in cell biology. Chapter 3. Unit 3.41:1-9.
12. Dentler, W.L., LeCluyse, E.L. (1982). The effects of structures attached to the tips of tracheal ciliary microtubules on the nucleation of microtubule assembly in vitro. Cell Motility. 2: 13-18.
13. Dentler, W. L., Rosenbaum, J. L. (1997). Flagellar Elongation and Shortening in *Chlamydomonas*. III. Structures Attached to the Tips of Flagellar Microtubules and Their Relationship to the Directionality of Flagellar Microtubule Assembly. KU ScholarWorks, Rockefeller University Press. 74: 747-759.
14. Emmer, B.T., Maric, D., Engman, D.M. (2010). Molecular mechanisms of protein and lipid targeting to ciliary membranes. Journal of Cell Science. 123: 529-536.
15. Gibbons, I.R., Rowe, A.J. (1965). Dynein: a protein with adenosine triphosphatase activity from cilia. Science. 149:424-426.

16. González-Ballester, D., de Montaigu, A., Galván, A., Fernández, E. (2005). Restriction enzyme site-directed amplification PCR: a tool to identify regions flanking a marker DNA. Anal Biochem. 15; 353(2):302.
17. Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., Rokhsar, D.S. (2011). Phytozome: a comparative platform for green plant genomics. Nucleic acids research. 40: D1178-86.
18. Gorman, D.S., Levine, R.P. (1965). Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardii*. Proc. Natl. Acad. Sci. USA. 54: 1665–1669.
19. Gupta, A., Diener, D.R., Sivadas, P., Rosenbaum, J.L., Yang, P. (2012). The Versatile Molecular Complex Component LC8 Promotes Several Distinct Steps of Flagellar Assembly. The Journal of Cell Biology. 198(1):115-126.
20. Inadome, H., Noda Y., Kamimura, Y., Adachi. H., Yoda, K. (2007). Tvp38, Tvp23, Tvp18 and Tvp15: novel membrane proteins in the Tlg2-containing Golgi/endosome compartments of Saccharomyces cerevisiae. Exp Cell Res. 313(4):688-97.
21. Keller, R., Schneider, D. (2013). Homologs of the yeast Tvp38 vesicle-associated protein are conserved in chloroplasts and cyanobacteria. Frontiers in Plant Science. 4:467.
22. Kim, H., Kim, T., Jeong, B-C., Cho, T-II., Han, D., Takegahara, N., Negishi-Koga, T., Takayanagi, H., Lee, J.H., Sul, J-Y., et al. (2013). Tmem64 modulates calcium signaling during RANKL-mediated osteoclast differentiation. Cell Metab. 17(2):249-60.
23. Kim, S., Zaghloul, N.A., Bubenshchikova, E., Oh, E.C., Rankin, S., Katsanis, N., Obara, T., Tsiokas, L. (2011). Nde1-mediated inhibition of ciliogenesis affects cell cycle re-entry. Nature Cell Biology.13: 351–360.
24. King, S.M., Patel-King, R.S., Wilkerson, C.G., Witman, G.B. (1995). The 78,000-M(r) intermediate chain of *Chlamydomonas* outer arm dynein is a microtubule-binding protein. The Journal of cell biology. 131(2): 399-409.
25. King, S.M., Witman, G.B. (1988). Structure of the gamma heavy chain of the outer arm dynein from *Chlamydomonas* flagella. The Journal of cell biology. 107(5): 1799-808.
26. Kong, J.N., Hardin, K., Dinkins, M., Wang, G., He, Q., Mujadzic, T., Zhu, G., Bielawski, J., Spassieva, S., Bieberich, E. (2015). Regulation of Chlamydomonas flagella and ependymal cell motile cilia by ceramide-mediated translocation of GSK3. Molecular biology of the cell. 26(24): 4451-65.
27. Lechtreck, K-F., Gould, T.J., Witman, G.B. (2013). Flagellar central pair assembly in *Chlamydomonas reinhardtii*. Cilia 2:15.
28. Li, L., Tian, G., Peng, H., Meng, D., Wang, L., Hu, X., Tian, C., He, M., Zhou, J., Chen, L., et al. (2018). New Class of Transcription Factors Controls Flagellar Assembly by Recruiting RNA polymerase II in *Chlamydomonas*. Proceedings of the National Academy of Sciences of the United States of America., U.S. National Library of Medicine. 115(17):4435-4440.
29. Li, X., Zhang, R., Patena, W., Gang, S.S., Blum, S.R., Ivanova, N., Yue, R., Robertson, J. M., Lefebvre, P.A., Fitz-Gibbon, S. T., et al. (2016). An Indexed,

Mapped Mutant Library Enables Reverse Genetics Studies of Biological Processes in *Chlamydomonas reinhardtii*. The Plant cell. 28(2): 367-87.
30. Lindemann, C.B., Lesich, K.A. (2010). Flagellar and ciliary beating: the proven and the possible. J Cell Sci. 123:519–528.
31. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T.L. (2008). NCBI BLAST: a better web interface. Nucleic Acids Research. 36(2): W5–W9.
32. Martin-Tryon, E.L., Harmer, S.L. (2008). XAP5 CIRCADIAN TIMEKEEPER coordinates light signals for proper timing of photomorphogenesis and the circadian clock in Arabidopsis. Plant Cell. 20:1244–1259.
33. Mayr, B., Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nature Reviews Molecular Cell Biology. 2: 599–609.
34. McCallion, P., De Soyza, A. (2017). Cough and bronchiectasis. Pulmonary Pharmacology & Therapeutics. 47: 77-83.
35. Meslet-CladiÃ¨re, L., Vallon, O. (2011). Novel Shuttle Markers for Nuclear Transformation of the Green Alga *Chlamydomonas Reinhardtii*. Eukaryotic Cell. 10(12):1670–1678.
36. NCBI Co. 2016. Database resources of the National Center for Biotechnology Information. Current neurology and neuroscience reports. Nucleic Acids Res. 2015; 44(D1): D7-19.
37. Pazour, G.J., Agrin, N., Leszyk, J., Witman, G.B. (2005). Proteomic analysis of a eukaryotic cilium. J. Cell Biol. 170:103–113.
38. Pazour, G.J., Witman, G.B. (2002). Forward and reverse genetic analysis of microtubule motors in *Chlamydomonas*. Methods. 22(4):285–98.
39. Pazour, G.J., Wilkerson, C.G., Witman, G.B. (1998). A Dynein Light Chain Is Essential for the Retrograde Particle Movement of Intraflagellar Transport (IFT). The Journal of Cell Biology. 141(4): 979–992.
40. Pitiakoudis, M., Tsaroucha, A.K., Katotomichelakis, M., Polychronidis, A., Simopoulos, C. (2005). Laparoscopic cholecystectomy in a patient with situs inversus using ultrasonically activated coagulating scissors. Histopathology. Acta Chir Belg. 105:114–117.
41. Swoboda, P., Adler, H.T., Thomas, J.H. (2002). The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in *C. elegans*. Mol Cell. 2000; 5:411–421.
42. Tucker, R.W., Scher, C.D., Stiles, C.D. (1979). Centriole deciliation associated with the early response of 3T3 cells to growth factors but not to SV40. Cell. 18(4): 1065-1072
43. Williams, J.C., Roulhac, P.L., Roy, A.G., Vallee, R.B., Fitzgerald, M.C., Hendrickson, W.A. (2007). Structural and thermodynamic characterization of a cytoplasmic dynein light chain-intermediate chain complex. Proc. Natl. Acad. Sci. USA. 104:10028–10033.
44. Yu, T., Laird, J.R., Prescher, J.A., Thorpe, C. (2018). *Gaussia Princeps* Luciferase: A Bioluminescent Substrate for Oxidative Protein Folding. Protein Science. 27(8):1509-1517.