Characterization of Three Sexually Sterile Mutants of the Green Alga *Chlamydomonas reinhardtii*

By
Condred Roberts

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Texas Tech University
Lubbock, Texas

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Introduction

The unicellular, biflagellated green alga *Chlamydomonas reinhardtii* is capable of reproducing both sexually and asexually. Under normal environmental conditions, cells exist as single haploid cells (vegetative cells) that reproduce asexually by mitosis. When environmental conditions change, vegetative cells switch to a sexual mode of reproduction. To initiate sexual reproduction, vegetative cells undergo gametogenesis, during which the cells activate a collection of gamete-specific genes (Ferris and Goodenough 1997). Gametogenesis results in the formation of one of two types of gametes termed mating type plus (mt+) and mating type minus (mt-). (Ferris, Armbrust et al. 2002) The gamete type produced is dependent on sex-specific genes present in the genome of the parent vegetative cell (Ferris and Goodenough 1997). The two haploid gamete types fuse forming one diploid zygote. The diploid zygote ultimately divides by meiosis and germinates releasing haploid vegetative cells.

Under laboratory conditions, vegetative cells of a single mating type are propagated by asexual mitotic divisions under a 14:10 light:dark cycle in nitrogen-rich media. To induce gametogenesis, the vegetative cells are placed in a nitrogen-free medium and exposed to continuous light. These cells undergo a gametogenic cell division that produces gametes. Subsequent mixing of mt+ and mt- gametes brings about the complex process of fertilization.

Fertilization in *C. reinhardtii* has many processes and steps analogous to those of other organisms, ranging from human fertilization to viral fusion (Wilson and Snell 1998). The similarities to processes in the viral life cycle illustrate that *Chlamydomonas* fertilization can be used to study processes other than just fertilization. With fertilization being the first

step in creating a life, it is an important process and could be used to control the spread of disease, help provide safe and less permanent forms of prophylaxis, or possibly even control the spread of invasive species. With many stages, made up of many signaling cascades and cellular processes, fertilization is controlled by a large number of genes, many of which have not been assigned a role in fertilization. Fertilization can therefore be controlled in a large number of ways but until the aforementioned genes are identified the process will continue to go on, unhindered.

Chlamydomonas as a Model Organism

Chlamydomonas is an excellent example of a model organism for a number of reasons. The entire *Chlamydomonas* genome has been sequenced and was most recently updated in March, 2009 and is available at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html. With the entire genome sequence known, *Chlamydomonas* is an excellent candidate for studies in genetic analysis. Moreover, the genome is rather small when compared to some model organisms, but is still large enough so that its size is not restrictive (table 1).

Table 1. Genomic Comparison Among Model Eukaryotic Organisms

Species	Genome Size (MB)	Estimated Gene Number	Chromosome Number
Chlamydomonas reinhardtii	100	15,256	17
Saccharomyces cerevisiae	12	5,800	16
Arabidopsis thaliana	125	31,407	5
Caenorhabditis elegans	100	20,000	6
Drosophila melanogaster	180	13,600	7
Homo Sapiens	3200	32,000	23

The *Chlamydomonas* life cycle is another very important factor in making *Chlamydomonas* a model organism, especially for fertilization studies. *Chlamydomonas* are

easy to culture in regular lab conditions. Grown in liquid culture, *Chlamydomonas* can be ready for use in experiments in as little as 3-4 days. *Chlamydomonas* spends the vast majority of its life in an, asexually reproducing, vegetative phase. Maintained in nitrogen-rich media on a 14:10 light/dark cycle, these cells will continue to reproduce asexually. Gametogenesis is easily induced by depletion of nitrogen and placing the cells under light for an extended period of time (approx. 12 hours). Sexual reproduction can only begin once gametes of different types, mt+ and mt-, are mixed together. Fertilization can thus be controlled and very easily studied.

A key tool in understanding cellular function in many model organisms including *Chlamydomonas* is the use of mutant strains. Numerous mutant strains defective at various reproductive stages have been created. A logistical problem typically encountered when working with sterile mutants is the inability to propagate subsequent mutant individuals. For example, heterozygous mice must be maintained, mating, and the subsequent offspring screened for homozygous offspring with the sterile allele. Propagation of sterile mutants is not a problem in *Chlamydomonas*. The alternating asexual and sexual modes of reproduction allows for the generation of mutants defective in sexual reproduction that can the propagated by asexual reproduction.

Genetics of Fertilization

Some of the more important fertilization mutants in *Chlamydomonas* are the Fus1, Hap2, Imp11, Sad1, Gam mutants. The *Sad1* mutants are mt- mutants that are defective in the production of mt- agglutinins (Hwang et al., 1981; Ferris et al., 2005). These gametes undergo normal gametogenesis but fail to agglutinate with mt+ gametes. These mutants will respond to cAMP by losing their cell walls exposing their mating structures; however,

fertilization rates are extremely low due to the lack of agglutination. Physically forcing cells into close proximately to cAMP-activated mt+ gametes by centrifugation will increase fertilization success. Together, this suggests that the Sad1 mutants are defective solely in flagellar agglutinins and aside from generating the cAMP signaling pathway agglutination is essential of physically positioning gametes to allow mating structure adhesion and fusion.

The Fus1 mutant is deficient in its FUS gene and is unable to adhere by its mating structure to the activated mating structure of an mt- gamete (Misamore et al., 2003). It is unknown whether the FUS gene is involved in subsequent gamete fusion or only the initial mating structure adhesion.

The Imp11 mutant is a MID mutant that is rather interesting (Ferris and Goodenough, 1997). This mutant is mt- but has a defective MID gene that keeps it from expressing its mt- fertilization genes. However, the FUS gene is not expressed, so the gametes behave as mt+ gametes but cannot fuse with mt- gametes. This means the Imp 11 gametes will agglutinate with wt mt- gametes and will activate fertilization tubules, but cannot adhere to the wt mt- gametes by their mating structure because they are FUS deficient.

Another mutant with severely-impaired ability to undergo sexual reproduction is the Ida5 mutant (Kato-Minoura et al., 1997). The Ida5 mutant is deficient in a gene coding for *Chlamydomonas* actin. Since actin is necessary for elongation of fertilization tubules, these mutants do not form normal fertilization tubules. When activated, the fertilization "tubule" is a small bulbous structure less than 1 μm in size. These mutants exhibit minimal to no ability to undergo fertilization. The Ida5 mutant is also deficient in its ability to localize the Fus1 protein to its mating structure (Misamore, personal communication).

There are two fusion-defective mutants in *Chlamydomonas*. The Gam mutants are temperature-sensitive mutants mt- mutants that undergo mating structure adhesion but not fusion at the restrictive temperature (Forest, 1983). Little beyond the phenotype of this mutant is known. The recently generated Hap2 mt- mutant is lacking a protein essential for mating structure adhesion. Hap2 mutants are able to adhere via their mating structures with mt+ gametes but do not undergo mating structure fusion. The HAP2 gene is a conserved gene that expresses a cell fusion protein. Other HAP2 genes with similar functions have since been described in other organisms including *Plasmodium* (Liu et al, 2008).

Fertilization Overview

As mentioned, vegetative cells must undergo gametogenesis to enter a sexually reproductive phase. One step in the gametogenic process is the expression and isolation of cell adhesion molecules located on the flagella of gametes. These adhesion molecules, called agglutinins, are mating-type specific and bind to complementary agglutinin molecules of the opposite mating type. Upon mixing mating types, the gametes adhere via their complementary flagellar agglutinins and form clumps of cells in a stage called agglutination. Adhesion of flagellar agglutinins induces a signaling cascade resulting in gamete activation.

Gamete activation is a combination of developmental events that prepares the gametes for cell binding and fusion. A major component of gamete activation is the production of the intracellular signaling molecule cAMP. cAMP has been shown to induce a number of downstream gamete activation events (Pasquale and Goodenough, 1987). One gamete activation event is the continued expression and transport of additional agglutinin molecules from the cell body onto the flagella (Goodenough, 1989). Another activation event is the release of an enzyme (lysin) that leads to the loss of the cell walls enabling cell

membranes to fuse (Buchanan et al., 1989). A critical cAMP-dependent gamete activation event is the activation of mating structures on both mt+ and mt- gametes (Wilson et al 1997). Mating structures are specialized regions of the cell surface where cell-cell adhesion and fusion will occur. These mating structures must be activated for fertilization to occur. After cell-cell fusion and the subsequent formation of a quadriflagellated diploid zygote, the flagella lose their adhesiveness and begin to beat in coordination, allowing for motility of the newly created zygote (Snell 1976). While many of the morphological stages of fertilization are known, the genes, gene products, and regulatory mechanisms involved in each step are relatively unknown. Until those genes and mechanisms are identified, it will be impossible to clearly understand this intricate process.

Agglutination

The first step in the fertilization process is agglutination, in which large clumps of mt+ and mt- gametes form a mass by binding to one another by their flagella. This binding is mediated by a flagellar protein called agglutinin. (Ferris, Waffenschmidt et al. 2005). Agglutinin is approximately 228 nm in length with a 10 nm head and a 218 nm shaft that is curved at its proximal end to create a "hook." This molecule is recruited to the membrane of the *Chlamydomonas* flagella during gametogenesis (Goodenough, Adair et al. 1985). While similarities such as size and structure exist between mt+ and mt- agglutinins, each agglutinin type is encoded by a separate gene (Ferris, Waffenschmidt, et al. 2005). Each mating type has genes for both agglutinin types, but only one specific gene is expressed.

Once two mating types bind by their flagella, additional agglutinin molecules are recruited from the cell body to the flagella where they become active (adhesive) (Goodenough 1993). If the agglutinin recruit to the flagella is inhibited, all flagellar adhesion

will be lost due to agglutinin recycling. Over time, the clumps of agglutinating cells decrease in size as the number of cells attached to one another is reduced so that only one mt+ gamete is attached to each mt- gamete. This represents the end of agglutination and the beginning of the next stage of fertilization, mating structure adhesion.

The agglutination stage of fertilization sets in motion a cell-signaling cascade that leads to gamete activation. Without these gametes becoming activated, all other stages of fertilization would not come to fruition. The first signal is the generation of gamete-specific adenylyl cyclase causing the aforementioned intracellular rise in cAMP (Pan and Snell, 2000). The generation of cAMP induces several downstream events essential for gamete activation. The steps of gamete activation are described below, but would not be possible without the early generation of adenylyl cyclase during agglutination. The blocking of any stage of this signaling pathway would more than likely stop the fertilization process immediately.

Cell Wall Loss

Some time after agglutination, the cells lose their walls (Pan and Snell, 2000). Flagellar adhesion induces the sexual signal leading to a rise in intracellular cAMP levels. Increased cAMP induces the release of lysin, the enzyme responsible for cell wall loss and degradation (Buchanan et al., 1989). Cell wall loss (CWL) exposes the underlying cell membrane and specifically the mating structures where cell-cell adhesion and fusion occurs. Gametes that have not lost their cell wall are unable to fuse. Cell wall loss is also accompanied by the activation of the mating structures. It is not clear whether activation of mating structures occurs before or after the cell wall is removed (Snell, 1976).

Mating Structure Activation

Mating structures are specialized regions of the cell where cell-cell adhesion and fusion will occur. They are located at the apical end of the cell between and slightly dorsal to the two flagella. The unactivated mating structures of both mt+ and mt- gametes are indistinguishable at the light microscope level. At the electron microscopy level, the unactivated mt+ and mt- mating structure is characterized by an electron-dense structure just below the plasma membrane, called the membrane zone. Underlying the membrane zone of the mt+ gamete is another electron-dense region known as the doublet zone (Goodenough and Weiss, 1975).

After activation due to the cAMP elevation during gamete activation, the mt+ mating structure undergoes an elongation forming an approximately 3 μm long microvillus-like structure termed the fertilization tubule. Fertilization tubule elongation requires actin polymerization just below the surface of the membrane (Goodenough *et al.* 1982). It is this elongated fertilization tubule that is used to attach to the mt- mating structure and begin the process of cell fusion. Along the plasma membrane of the activated and unactivated mt+ mating structure is an extracellular glycocalyx called fringe that is involved in mating structure adhesion (Wilson et al. 1997).

Activated *mt–* gametes produce a less prominent, dome-shaped mating structure. While it lacks the electron-dense doublet zone of the mt+ mating structure, the unactivated mt- mating structure is similar to the unactivated mt+ mating structure in that it contains a membrane zone underlying the plasma membrane and the plasma membrane is lined with component fringe (Wilson *et al.*, 1997).

Mating Structure Adhesion & Fusion

The final stage in sexual reproduction in *Chlamydomonas reinhardtii* is the formation of a diploid zygote. This is brought about by the fusion of two gametes of opposite mating type. The process of gamete fusion begins with the adhesion of the mating structures. Fusion occurs so quickly in *Chlamydomonas* that it has been difficult to observe and study cell-cell adhesion (Misamore et al, 2003). As mentioned earlier, the ultrastructural component fringe of the mt+ fertilization tubule has been shown to be involved in mating structure adhesion. The cell-surface protein *fus1* has also been proven to play a major role in mating structure adhesion and fusion (Misamore et al, 2003). Immediately following mating structure adhesion, the cells come together in fusion and form a single, quadriflagellated, diploid zygote. This process of membrane fusion requires the HAP2 gene (Liu et al, 2008). This stage ends the fertilization process and leads to another complex process; zygote development.

Genetics of *Chlamydomonas* Reproduction

The genome is subdivided into 17 linkage groups. Along linkage group VI, there is a specific region called the mating type locus (Ferris and Goodenough, 1994). This region is the only known region of the *Chlamydomonas* genome that differs between mating types. A few genes have been identified in these loci. Located in the mt+ mating type locus is the FUS1 gene (Ferris and Goodenough, 1994). As mentioned, FUS1 encodes a cell-surface protein localized to the fertilization tubule that is essential for mating structure adhesion (Misamore et al., 2003). Within the mt- mating type locus is a regulatory gene (MID) (Ferris and Goodenough, 1997). Only a few hypothetical gene encoding regions have been proposed for the remainder of either mating type locus. Thus, most of the genes encoding

fertilization proteins including both agglutinins, structural proteins of both mating structures, and HAP2 are believed to be outside the mating locus. Therefore these fertilization genes would be present in both mating types. The regulation of their expression is dictated by the MID gene located in the mt- mating structure. If MID is present, mt- fertilization genes are expressed including mt- agglutinins, and mt- mating structure proteins. All mt+ fertilization genes are not expressed. Conversely, if MID is absent, the gametes express mt+ fertilization genes including mt+ agglutinins and the structural genes to assemble fertilization tubules (Ferris and Goodenough, 1997).

Objective

Recently, the Snell lab at UT-Southwestern Medical Center generated three additional mt+ mutants that are phenotypically unable to form zygotes (pfus2, pfus3, and pfus4). The goal of this project was to determine what steps in the fertilization process have been disrupted in each of these mutants. Additionally, we attempted to identify the specific genes disrupted in each of these mutants. Identifying exactly which stage of the fertilization process is dysfunctional in each of these mutants will be an important first step in determining the role of the disrupted genes. With all of the stages described above as candidates, the possibilities for phenotypes seem nearly endless.

To further increase the complexity, little is known at the genetic level about each stage of fertilization. While all of the steps of the process are understood in a broad sense, there is still a large amount of information that is simply not understood at this time. It is hopeful that once the phenotypes of each mutant are described that genetic analysis will lead to three different, and novel, genotypes. With three new genotypes of fusion mutants, three new points of control in the fertilization pathway could be identified. It is possible that these

mutants are simply new examples of previously defined mutants. This possibility is the reasoning behind the intricate detail that must be applied to each step of phenotypic and genotypic characterization.

The process began by comparing each mutant to 21gr wt mt+ gametes at each stage of the fertilization process, from gametogenesis to agglutination through fertilization tubule formation and ultimately mating structure adhesion. Cell fusion was not be observed as these mutants were selected for their inability to fuse and form zygotes. Whenever possible, cellular processes such as agglutinin-induced cell signaling were bypassed to determine if downstream events such as CWL and fertilization tubule formation still function.

Following phenotypic characterization, Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) and subsequent sequencing were attempted to allow for identification of the DNA flanking the insert and most likely the identification of the disrupted gene. Identification of the disrupted gene could then lead to a directed knock-out to strengthen the theory, and lastly, a rescue experiment in which the product of the gene of interest is added to the mutant to see if fertilization continues as it does in wild type gametes.

Materials and Methods

Cell Culture

C. reinhardtii strains 6145c (mt-), 21gr4 (mt+), pfus2 (mt+), pfus3 (mt+), and pfus4 (mt+) were maintained in liquid culture as vegetative, asexually reproducing, cells under constant aeration, at 22^{o} C on a 14:10 light/dark cycle in 150 ml M medum (Sager and Granick, 1954)**.** Cells were plated on 2% agar M medium and grown at 22^{o} C and subsequently stored at 12 ^{o} C for long term (2-3 months) use. For extended storage (~6 months) cells were stored on 2% agar M medium slants.

Gamete Induction

Vegetative cells are first transferred from 250 ml flasks to 250 ml round-bottom centrifuge tubes. Tubes are then placed in adapter sleeves and placed on a balance. The tubes were brought to equal weight by the addition of nitrogen free media (M-N). The cells werethen centrifuged at 3,000 rpm for 6 minutes at 4^{o}C. While tubes were spinning, original 250 ml flasks were rinsed once with DI water and once with M-N to remove any water that was still in the flask.

After centrifugation, the supernatant (the M media) was poured off and the pellet was resuspended in M-N. Upon resuspension, the solution was transferred back to the original flask, already washed with M-N. The flasks were then placed under constant light so as to deplete any remaining nitrogen. Gametes were ready for use in experiments within 10-12 hours.

Agglutination of Gametes

Chlamydomonas mt+ and mt- gametes were added by Pasteur pipette in equal volume to 1.5 ml conical bottom tubes. One drop of mixed cells was added to a microscope slide for observation under a microscope. Cells were checked for agglutination immediately upon mixing, and at 5, 15, and 30 minutes after mixing. Degree of agglutination was measured qualitatively using a "-, +, ++, +++" system in which "-" represented no agglutination and +++ represented 100% of cells agglutinated.

Activation of Gametes

Prior to activation, gametes were screened by a photoselective process to concentrate the highest quality gametes. The gametes were placed in a 250 ml graduated cylinder in front of a fluorescent light. In response to the high light intensity, the gametes were allowed to

swim to the bottom of the cylinder for ~10 minutes. All but the bottom 30 ml of fluid in the cylinder was siphoned off and discarded. The cells that were unable to respond to the light stimulus and were unable to swim to the bottom were discarded.

This photoselection selects for the active gametes, the concentrated cells that were strong swimmers and able to respond to external stimuli. These gametes were then transferred to 50 ml round bottom tubes and allowed to swim down further for ~5 minutes to further concentrate the cells. 0.5 ml of gametes was taken from the darkest green region at the bottom of the tube and transferred to either a 50 ml conical bottom tube or 1.5 ml microcentrifuge tube.

Activation of gametes was achieved in two methods. The native method involved mixing cells of opposite mating types. Qualitative measurement of activation of gametes, by mixing of mating types, was carried out by mixing 500 μl aliquots of equal cell volume of photoselected mt+ and mt- cells into a 1.5 ml microcentrifuge tube. The second method of gamete activation involved incubating the cells in dibutryl cAMP. This was achieved by mixing 5 μl of Papaverine and 50 μl of dbcAMP, which were added to 500 μl of photoselected mt+ gametes in a 50 ml conical bottom tube. Papaverine served to keep the dbcAMP from breaking down. Gametes, dbcAMP, and Papaverine were aerated on high bubble rate for 30-60 minutes. Cells were subsequently checked for cell wall loss.

Assaying Cell Activation

I. Cell Wall Loss

Cell wall loss (CWL) was measured qualitatively. Preparation was performed by adding 10 μl of activated cells to 1 ml of cell wall lysis buffer and vortexing for 10 seconds. The mixture was allowed to sit for 10 seconds before centrifugation at 8000 rpm for 30

seconds. Qualitative measurement of CWL was done using a "-, +, ++, +++" system in which "-" represents no CWL and "+++" represented 100% CWL. Cells were allowed to mix for 10 minutes and then checked for cell wall loss

To measure CWL, a number of assays can be performed. The two simplest and most effective ways were used in this study. First, gametes of opposite mating type were mixed together in equal cell volumes and CWL was measured using a CWL lysis buffer at 15 and 30 minutes. Taking a measure of intracellular adenylyl cyclase or cAMP would be the most exact way to determine whether or not cells were producing enough of these molecules, or if there was perhaps a signaling pathway deficiency. However, the more efficient method of detecting a cell signaling deficiency is to add dibutryl cAMP to gametes to induce activation. Following activation, cells with a properly functioning signaling pathway will have CWL similar to that of the cells mixed with opposite mt. These two assays will allow for measurement of CWL (and later, FT formation) and rule out, or confirm, a signaling pathway deficiency.

II. Fertilization tube formation

The cells were stained with Phallocidin for fluorescence microscopy and checked for tubes. This method allowed for quantitative measurement of fertilization tube activation if equal amounts of cells were used in each sample.

Phallocidin Staining and Fluorescence Microscopy

Activated mt+ gametes were prepared for fluorescence microscopy by phallocidin staining on 8 well slides. Each well to be used was coated with 10 µl 0.1% polyethylenimine (PEE) diluted 1:1 with DI H_2O. The wells were allowed to dry for approximately 5 minutes and the excess PEE was siphoned off. Five ml of activated gametes were added to each well

and allowed to dry for 10-20 minutes under a hood and then acetone treated at 80% for 6 minutes and then 100% for 6 minutes. The slides were allowed to air dry for 1-2 minutes and then 20 μl of diluted phallocidin stain was added to each well and the slides were put into a humidity box at 37^{o} C for 30 minutes. After incubation in the humidity box the slide was rinsed in phallocidin buffer in a staining jar for 6 minutes. Fluoromount G mounting media was added to slide and a 24x60 No. 0 cover slip was placed on the slide and sealed with nail polish.

Fertilization tubule activation was measured qualitatively and quantitatively by observation on a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., New York, NY) containing FITC/GFP and TRITC/Rhodamine barrier filters. Fertilization tubule activation was scored qualitatively using a "-, +, ++, +++" system in which "-" represents no fertilization tubule and "+++" represented 100% fertilization tubule activation. Fertilization tubule activation was scored quantitatively by recording the percentage of gametes with fertilization tubule in five fields of view.

DNA Extraction

DNA for PCR and other genetic analysis was extracted and isolated using the protocol described by Doyle *et al.* (1990). Two tubes containing 25 ml of cells were spun at 3000 rpm for 5 minutes in a screw cap conical bottom tube. One tube of cells was resuspended in 15 ml M-N medium and added to the pellet of the second tube. The remaining volume of the screw cap conical bottom tube was then filled with M-N medium and spun down at 3000 rpm for 10 minutes. The supernatant was siphoned off and the pellet resuspended in 1 ml of heated CTAB buffer and added to a 1.5 ml microcentrifuge tube to be shaken at 60^{o} for 1 hour. Phenol/chloroform/isoamyl alcohol extraction (25 parts phenol, 24

parts chloroform, 1 part isoamyl alcohol) was performed twice and the DNA was subsequently precipitated spinning for 15 minutes at 13,000 rpm in 2 volumes of an EtOH/NaCl mixture (final NaCl concentration 0.2 M). The pellet was then washed twice with 70% EtOH and 2 μl of RNase A was added to the mixture and phenol/chloroform/isoamyl alcohol extraction performed once again. DNA was precipitated out by spinning for 15 minutes at 13,000 rpm and pellet was washed with 70% EtOH twice. Pellet was resuspended in 50 ml TE buffer (pH 8).

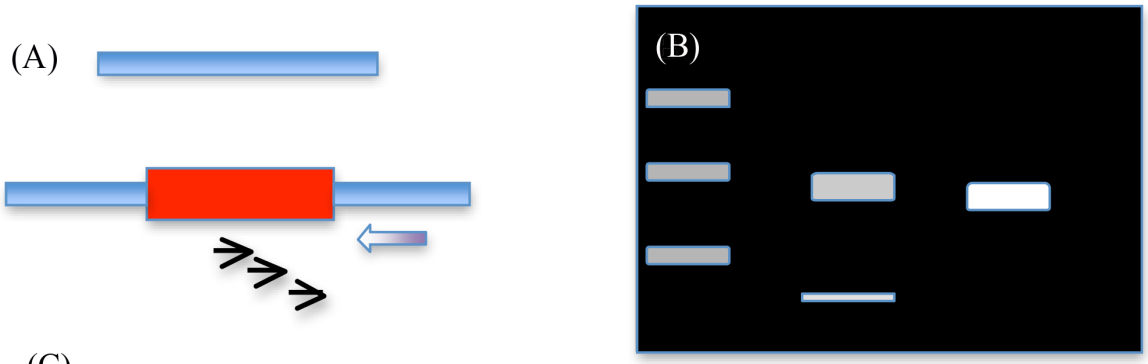
TAIL-PCR

Isolating the location of the insert causing the mutation in the mutants was achieved by using Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) as described by Singer (2003). This particular method of PCR uses a degenerate primer of 64 fold degeneracy against 4 nested primers. The intent of this process is to amplify a small piece of DNA flanking the insert for genetic sequencing and analysis to determine the location of the insert. The initial PCR will amplify a piece of DNA including the insert and the area flanking it (Fig. 1). However, multiple banding patterns may occur due to the degeneracy of the primer. The subsequent 3 PCR runs should amplify and the original band with the insert and flanking DNA and eliminate the multiple bands. The final product can then be extracted from a gel after electrophoresis and transformed into competent cells in order to clone the single sequence flanking the insert.

Transformation and Cloning

Cloning and amplification of the TAIL-PCR product was carried out by transforming the product into competent DH5α plasmids. To begin the process an agarose gel with each of the four TAIL-PCR products was run to confirm that the process had amplified one piece

of DNA and not multiple bands. Next a gel was run using the remaining 22 µl of the fourth product. A gel extraction was performed using a QIA Quick Extraction Kit (Qiagen, Valencia, CA) and the protocol provided by the kit.



(C)

AP1
5'-(G/C)TA GA(G/C) T(G/C)T (G/C)G(A/T) GT(G/C)-3'
pSI103-F2
5'- CCC ACG AGG AGG ACC CGT GGT T -3'
pSI103-F3
5'- TGG TTC GGG CCG GAG TGT TCC -3'
pSI102-F4
5'- CGG TAT CGG AGG AAA AGC TGG CG -3'
pSI103-F8
5'- GTC GCG TGG GTG ACA GCA CAG TG -3'

Figure 1 Shows the basic concept of TAIL-PCR. (A) The three black arrows represent the nested primers designed to match the insert, while the left-pointing arrow represents the degenerate primer, designed to match multiple areas of the genome. (B) This illustration represents an ideal gel electrophoresis of a TAIL-PCR sample. The first lane shows multiple bands due to the amplification of multiple sites by the degenerate primer and the first nested primer. The second lane still shows multiple bands due to the degeneracy of the primer, but also shows a slight amplification of one of the bands from the first lane. The third lane shows only one band, an amplification of the band from the first and second lanes. This DNA fragment is slightly smaller (in base pairs) than the fragments in the first and second lane, but is much brighter and larger in band size. This piece of DNA can then be extracted from the gel and used later for sequencing. It is not uncommon to use a fourth TAIL-PCR run to further amplify the piece of flanking DNA. (C) Sequence of degenerate (AP1) and sequential nested primers (pSI103-F2, F3, F4, F8)

The DNA product's ligation was performed by mixing 5 µl of a 2x ligation buffer, 1 µl of T vector, 1 µl of T4 ligase, and 3 µl of the DNA sample and incubating for 1 hour at RT. After one hour, 50 µl of competent DH5α plasmids was mixed with 5 µl of ligated DNA and incubated on ice for 30 minutes. The mixture was heat shocked at 42^{o} C for 90

seconds and then again placed on ice for another 2 minutes. After 2 minutes on ice 0.15 ml of S.O.C medium was added and the mixture was placed on a shaker at 225 rpm at 37 ^{o} C for 1 hour. Cells were then plated on LB +amp plates at 37^{o} C overnight. After incubation overnight the plates were stored at 4° C to prevent further growth. Ten colonies were picked from the plate using a micropipette tip and placed in 3 ml of LB +amp liquid medium. The liquid cultures were then placed in a shaker at 225 rpm at 37^{o} C overnight.

After shaking overnight DNA was isolated from the broth using the protocol provided by the Qiagen Miniprep QuickSpin Kit (250) (Qiagen, Valencia, CA). Once DNA was isolated, it was digested in a mixture of 2 μl EcoRI, 10 μl NE labs 10x EcoRI buffer, 10 μl DNA, and 78 μl DI water, for one hour. After one hour of incubation, gel electrophoresis revealed whether or not the DNA fragment was taken up by the DH5α competent cells.

Sequencing

Sequencing for TAIL-PCR products was performed using two separate methods during this project. Sequences obtained for pfus3 and pfus4 were transformed and cloned and sent to the DNA Sequencing Core Facility at UT Southwestern Medical Center, Dallas, TX. Sequences for pfus2 were also sent for sequencing at the sequencing center, as well as sequenced at TCU using a general capillary electrophoresis sequencing protocol.

After TAIL-PCR products were run on a gel to confirm amplification of one piece of DNA, a gel extraction was performed using the protocol provided by the Wizard SV Gel Clean-Up System (Promega Corporation, Madison, WI). After DNA extraction, the DNA sample was prepared for sequencing.

Deflagellation

Deflagellation and the subsequent reflagellation of gametes was measured under a microscope and cells were qualitatively measured using a "-, +, ++, +++" system in which "-" represents no deflagellation or reflagellation and "+++" represents complete deflagellation or reflagellation. Gametes were photoselected and transferred to 0.5 L bottles and spun at 3,000 rpm for 15 minutes. Just enough M-N medium was added to each bottle to resuspend the pellet. Gametes were pH shocked with acetic acid to pH 4.4-4.5. Gametes were allowed to stay at pH 4.4/5 for 20 s – 1 min. After 1 minute, the pH was increased to 7.2 by addition of KOH. pH shocked cells (1 ml) were transferred to 1.5 ml microcentrifuge tubes and were subsequently monitored qualitatively for deflagellation and reflagellation under a microscope at 10x magnification at 5, 10, 15, 20 minutes and every 10 minutes after until 1 hour. At each observation, a measurement of deflagellated cells and reflagellated cells was recorded.

Microscopy

All light microscopy was carried out using a Zeiss Axiostar Plus microscope (Carl Zeiss Inc., New York, NY) paired with a Zeiss AxioCam MRm camera. Fluorescence microscopy was performed using the Zeiss Axiovert 200 microscope (Carl Zeiss Inc., New York, NY) containing FITC/GFP and TRITC/Rhodamine barrier filters mentioned previously. The fluorescent microscope was also coupled with a Zeiss Axiocam MRm camera.

Cell Counts

Cell counts were performed at 20X magnification on a light microscope, using a bright-line hemocytometer (Hausser Scientific, Horsham, PA). Cells were fixed by mixing 150 μl of photoselected gametes with 100 μl 5% gluteraldehyde fixative (Misamore et al,

2003) and 750 μl M-N medium in a 1.5 ml microcentrifuge tube. Cell counts were obtained by counting the number of gametes in 5 squares diagonally and multiplying the number of cells x 5 x 10^4 x dilution factor (6.66x) to obtain cells/ml.

Results

The initial objective of this study was to determine which events of *Chlamydomonas* fertilization are disrupted in the four mutant strains. I investigated several discernible stages in the fertilization process including agglutination, gamete activation, cell wall loss and fertilization tubule formation.

Agglutination

Mixing of wt- gametes with pfus2, pfus3, pfus4 and wild-type plus mutants (wt+) gametes yielded agglutination of differing degrees. The *Chlamydomonas* convention for assaying agglutination is a qualitative scaling system of +, ++, +++. Using this convention for assaying agglutination, I looked for differences in agglutination variability between the mutants and wt+ gametes. Figure 2 indicates the degrees of agglutination and the qualitative scores associated with them.

Agglutination was based on the standard *Chlamydomonas* convention for scoring relative amounts of agglutination. This system is utilized in part due to variable preservation of agglutinating cells during fixation. Thus, qualitative live-cell assays were performed. The system employs a -/+/++/+++ system. A (-) score indicates little/no agglutination. A (+) score indicates approximately 30-50% of the cells in the field of view are contained in agglutinating clumps. A score of (++) and (+++) indicate approximately 50-85% and 85-100% of cells agglutinating in clusters, respectively. At 5, 10, and 15 minutes, agglutination was present in all three mutant and wt+ gametes (Fig.3).

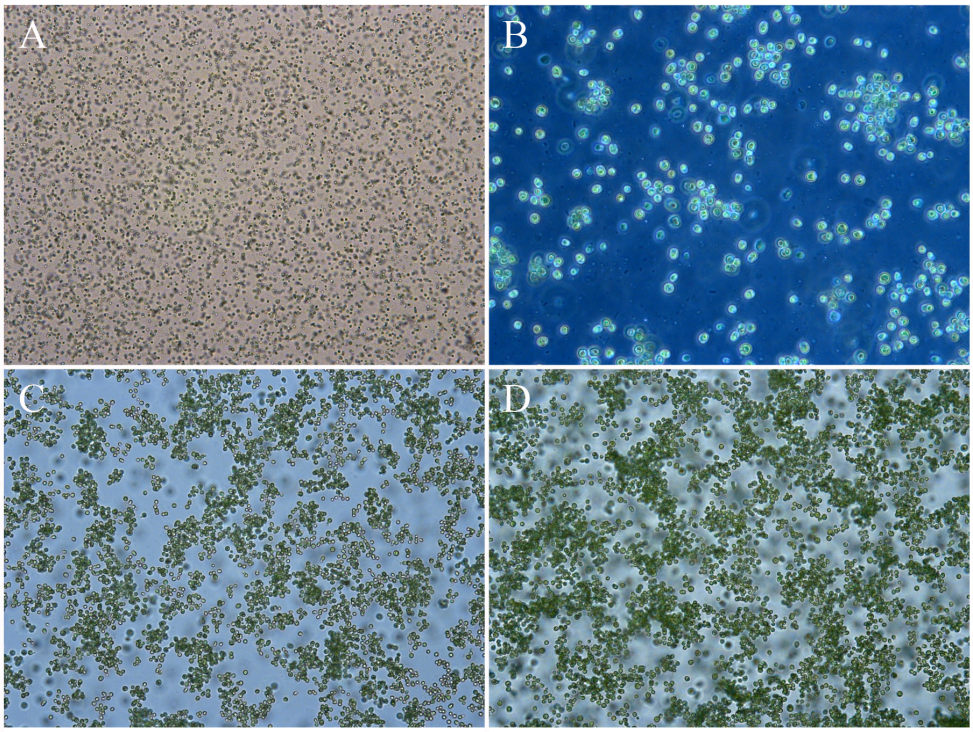


Figure 2. Brightfield micrograph of the qualitative measurement scale for of agglutination. (A) Few if any cells are agglutinating. Cell uniformly distributed throughout the field of view. Scored as (-). (B) A low percentage of cells are agglutinating but the vast majority of cells are not. Scored as (+). Magnification slighly higher to show limited agglutination. (C) The majority of cells are agglutinating. Scored as (++). (D) Nearly all cells are agglutinating. Scored as (+++).

The wildtype (wt+) gametes showed the highest degree of agglutination at every time point. All three mutants exhibited some level of agglutination at 5 min. By 10 min, all three mutants exhibited moderate to high (++, +++) levels of agglutination. There was a slight decrease in agglutination in pfus3 at 15 min relative to 10 min.

Deflagellation

To determine if there was a general disruption in signaling pathways or cellular assembly, the ability of *Chlamydomonas* to lose and subsequently reassemble new flagella due to stress was studied. Deflagellation and the subsequent reflagellation of

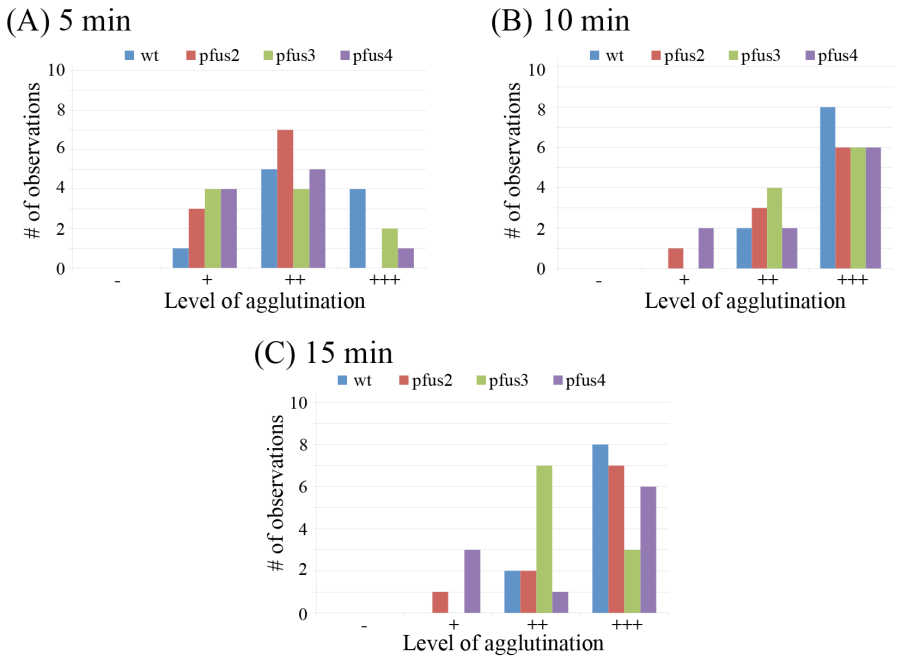


Figure 3. Qualitative results of gametes agglutinating at 5, 10, and 15 minutes. (A) At 5 minutes after mixing gametes, wild type and all three mutants exhibited agglutination. (B) At 10 minutes, the majority of each gamete type exhibited the highest level of agglutination (+++). (C) At 15 minutes, nearly all gametes types were agglutinating at the highest level except for pfus3. The Y axis represents the number of samples examined (N = 10).

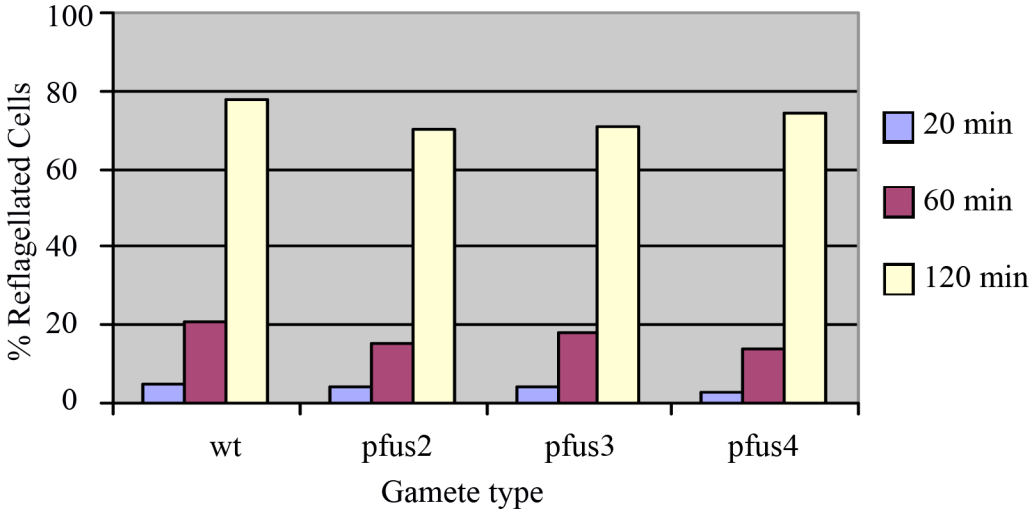


Figure 4. The ability of mt+ gametes to regenerate flagella as an indicator of general cell signaling function was tested. In wt+ gametes as well as all three mutants, gametes were able to regenerate flagella by 120 min after deflagellation.

Chlamydomonas mt+ gametes were measured. Qualitative and quantitative measurements of each type of cells were taken every 5 minutes for the first 30 min and every

30 min subsequent (Fig. 4). Cells began to show reflagellation as early as 10 min after deflagellation, but did not show any appreciable reflagellation until 45 min. While wt cells reflagellated at a faster rate, all three mutants showed complete reflagellation by 4 h.

Cell Wall Loss

The ability of wt- gametes to induce activation of mt+ gametes leading to mutual loss of cell walls was tested. Equal numbers of wt- gametes were mixed with either wt+ or one of the three mt+ mutants and were measured qualitatively at 10, 15, and 30 minutes. Levels of cell wall loss were based on the assay described in Figure 5. At 10 and 15 minutes after mixing, all three mutants and wt+ gametes showed slight activation and cell wall loss (Fig. 6). By 30 min, all three mutants as well as wt+ gametes showed high levels of cell wall loss when mixed with wt- gametes.

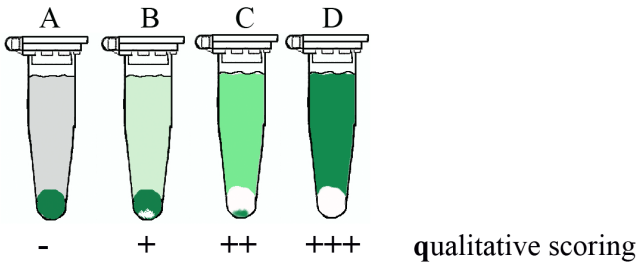


Figure 5. Illustrates the CWL assay qualitative results. (A) With no wall loss, all the chlorophyll remains in the intact cells. (B) With moderate wall loss, some chlorophyll is released in the supernatant (light green) and the pellet consists of intact (green) cells and cell fragments (white). (C) With high cell wall loss, most of the chlorophyll is released into the supernatant while he pellet consist of cell fragments. The pellet shows some intact, green cells. (D) Complete cell wall loss has ocurred with a completely white pellet. Qualitative scores of "-, +, ++, +++" are indicated.

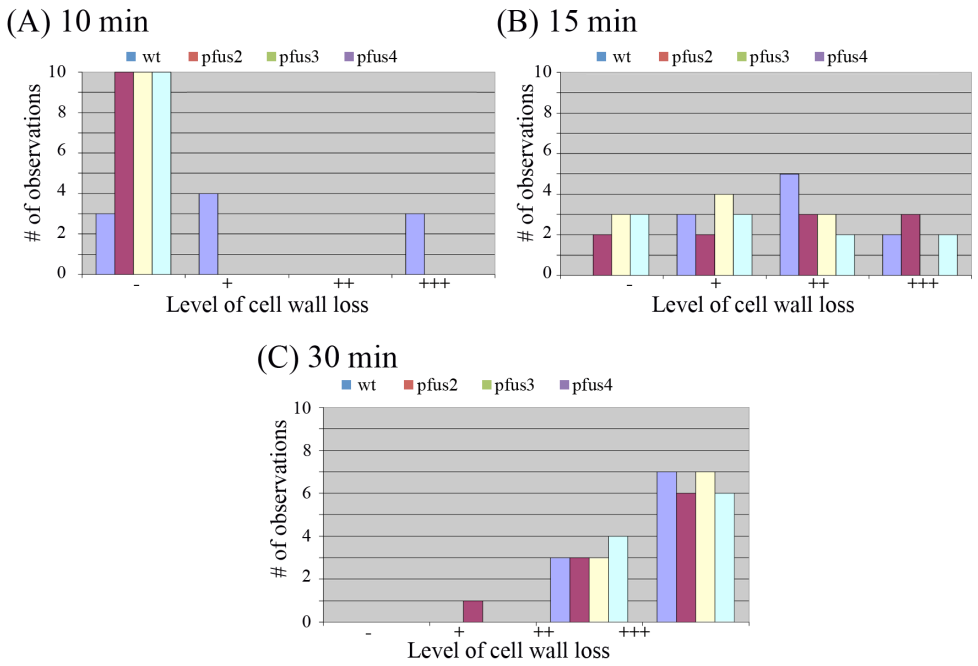


Figure 6. Gamete activation induced by mixing with wild type minus gametes. All three mutants and wild type plus controls were mixed with wt- gametes to determine agglutinin-induced cell wall loss. Cell wall loss was determined at 10, 15, and 30 minutes post mixing. (A) Cell wall loss after 10 min incubation in camp. Most samples showed little cell activation. (B) Cell wall loss after 15 min incubation in cAMP. (C) Cell wall loss after 30 min incubation in cAMP. Most samples from all three mutants and the wild-type plus gametes exhibitied cell acrtivation. Y axis indicated numbers of independent replicates. (N = 10).

To bypass possible defects in flagellar agglutination function, the ability to respond directly to increased levels of cell wall loss was examined (Fig. 7). When incubated in cAMP for 30 min, all three mutants and wt+ gametes exhibited high levels of gamete activation and cell wall loss.

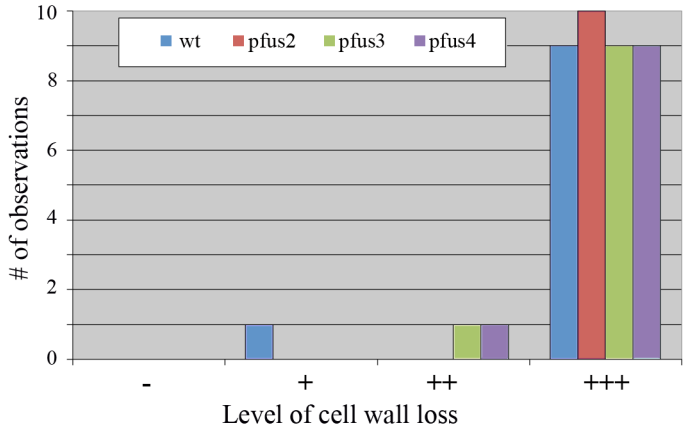


Figure 7. Wild type and the three mt+ mutants were evaluated for the ability to undergo activation and cell wall loss induced by cAMP. After 30 min in cAMP, both wild type and all three mutants exhibited high levels of cell wall loss. Y axis indicated numbers of independent replicates. (N=10).

Fertilization Tubule Formation

All three mutants were evaluated to determine if they were able to form activated mt+ mating structures (fertilization tubules). Mt+ gametes mixed with wild type mt- gametes to induce activation of both cell types. Mixed cells were fixed at 15 minutes and examined for presence of fertilization tubules by fluorescence microscopy. Fertilization tubules were observed in sample of wt+/wt- (Fig. 8A). In samples involving the mutants mixed with wt- gametes, no observable fertilization tubules were observed (Fig. 8B-D).

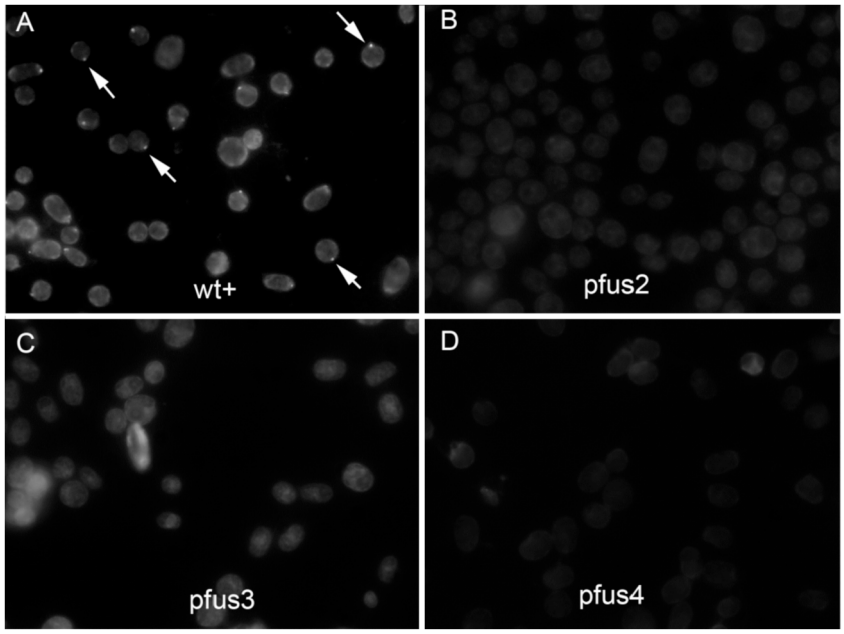


Figure 8. Wild type plus (mt+) and the sterile mutants were activated with cAMP and stained with phallocidin for the presence of fertilization tubules. Wt+ gametes (A) showed fertilization tubules while pfus2 (B), pfus3 (C), and pfus4 (D) failed to label with phallocidin.

To bypass agglutinin-signaling defects, fertilization tubule formation using cAMP was tested. To achieve this, cells were mixed with cAMP, fixed after 15 minutes, and percentage of cells with then counted under the microscope. Since all cells present are plus gametes, quantitative measurement of fertilization tubule formation can be determined. Total number of cells visible was counted under brightfield and number of fertilization tubule visible was counted under fluorescence. Five randomly selected fields of view a total of five independent replicates. Again, only 21gr gametes produced FT (Fig. 9). None of the mutants exhibited fertilization tubules. Figure 9 shows that an average of 56% of cells formed fertilization tubule in wt+ gametes after 15 minutes, while there was no fertilization tubule formation after 15 minutes for any of the mutant gametes.

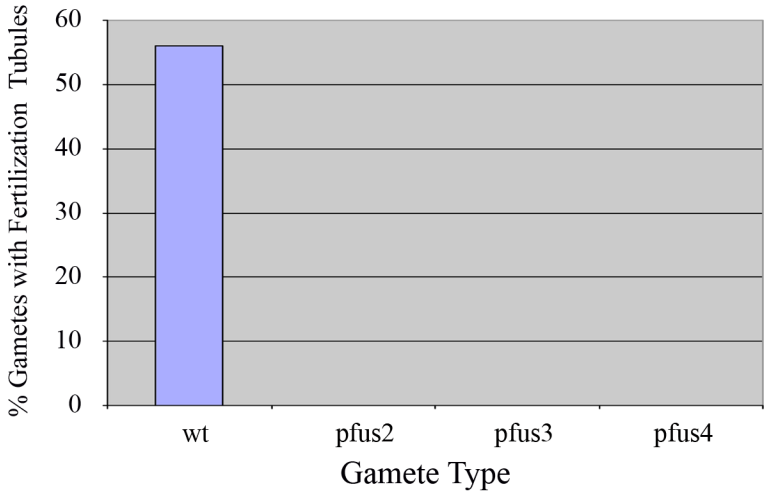


Figure 9. Wild type (wt) and the three mt+ mutants were activated with cAMP and examined for presence of fertilization tubules. Fertilization tubules were present on 56% of wt+ gametes, while pfus2, pfus3, and pfus4 showed no fertilization tubule formation.

TAIL-PCR and Sequencing

I. pfus2 Sequence Analysis

To identify the gene disrupted based on the position of the insertion, four sequential runs of TAIL-PCR using isolated genomic DNA from pfus2 as a template were performed. TAIL-PCR yielded the band approximately 300 kb shown in Figure 10. The band was excised and sent off for sequencing. Four attempts to sequence the PCR product directly failed to produce an interruptible sequence. Ligation of the PCR product into the cloning vector and subsequent transformation yielded one plasmid with the insert. EcoRI digestion of this isolated plasmid resulted in a band of appropriate size for the ligated PCR insert (Fig. 10). This plasmid was sent for sequencing and returned the sequence given in Figure 11. A BLAST against the *Chlamydomonas* genome revealed no hits for the sequence provided. The sequence BLASTN against all genome databases provided by the National Center for

Biotechnology Information (NCBI) showed that the only sequenced DNA belonged to the cloning vector.

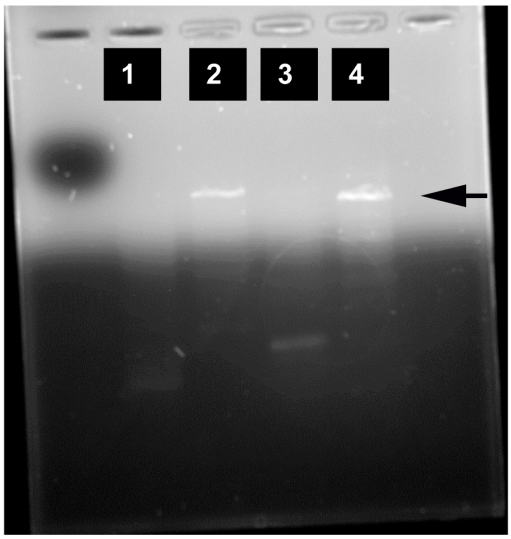


Figure 10. Tail PCR using 1 degenerate primer and three nested primers was performed. Lane 1 = first amplification using degenerate primer and nested primer 1. Lane 2 = PCR reaction using degenerate primer and nested primer 2 with PRC 1 product as template. Arrow shows fragment of interest. Lane 3 = third amplification using degenerate and nested primer 3 using second PCR product as template . Lane 4 = final amplification using degenerate and nested (4) primers using PCR product from third PCR reaction as template. Arrow indicates reappearance of the fragment from lane two. The band in the fourth well was excised and used for sequencing.

pfus2

CGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGC
ATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTC
ATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
CGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTA
ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCT
GTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTT
TGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGG
TTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGG
CCAGCAAAAGGNCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC
ATAGGCTCCGCCCCCCTGACGAGCATCACAAAANTCGACGCTCAAGTCAG
AGGTGNNGAAACCCGACAGGACTATNAAGATACCAGGNGTTNCCCCCTNG
ANGCTCCCTCGNGCGCTCTCNTGTTCCGACCCTGCCGCTTACCGGATACCT
GNCCGCCTTCNCCCTTCNGGAAGCGNGGNGCTTTCTCATAGCTNACGNTG

Figure 11. Sequence from cloning of TAIL-PCR product of pfus2.

The entire process from TAIL PCR, ligation and transformation, and analysis of resultant clones was repeated three additional times. All clones analyzed resulted in empty vectors and unsuccessful ligation of the TAIL PCR product. A sequencing run following the third attempt yielded the exact same sequence as the sequence shown in figure 11.

II. pfus3 Sequence Analysis

Genomic DNA from pfus3 was isolated and a TAILPCR performed the Snell lab (Fig. 12). This band was isolated, ligated into the cloning vector, and sequenced at UT-Southwestern (Fig. 13). Using the provided sequence I performed a nucleotide sequence BLAST. When comparisons were limited to the *Chlamydomonas* genome, the sequence seemed to fall in a non-coding region (Fig. 14). Two putative genes are projected on either side of the insert. One gene downstream of the insert encodes for a snare-like vesicle trafficking gene. The other gene located upstream of the insert encodes for a phosphoregulokinase.

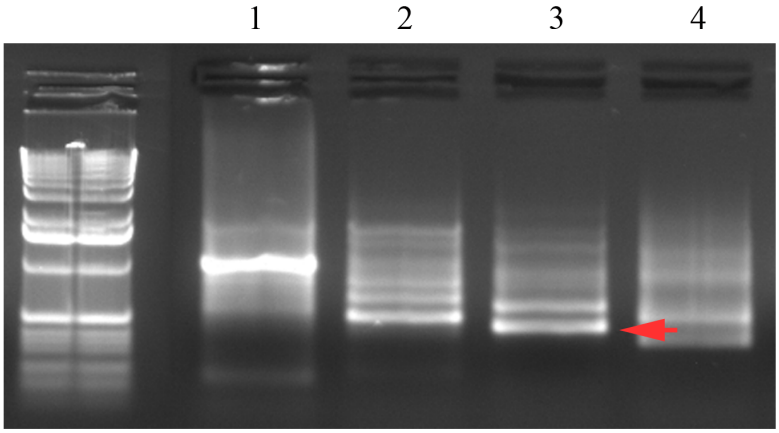


Figure 12. Tail PCR using 1 degenerate primer and three nested primers using fus3 genomic DNA was performed. Lane 1 = first amplification using degenerate primer and nested primer 1. Lane 2 = PCR reaction using degenerate primer and nested primer 2 with PRC 1 product as template. Lane 3 = third amplification using degenerate and nested primer 3 using second PCR product as template. Lane 4 = final amplification using degenerate and nested (4) primers using PCR product from third PCR reaction as template. Arrow indicates band in lane 3 that was ligated into the cloning vector and sequenced. Gel image provided by the Snell lab.

pfus3 sequence

TGGTGGCTGGGTA*GGTTGC*TCGCGTG**TGACAGCACAGTGTGGACGTT
GGGATCCCCGCTCCGTGTAAATGGAGGCGCTCGTTGATCTGAGCCTTGCCC
CCTGACGAACGGCGGTGGATG*AAGATACTGCTCTCAAGTGCTGAAGCGG
*AG*TT*TCTCCCCGTTTCGTGCTGATCAGTCTTTTTCAACACGTAAAAGCG
GAGGAGTTTTGCAATTTTGTTGGTTGTAACGATCCTCCGTTGATTTTGGCCT
CTTTCTCCATGCCTAAGTGCGCAAACAGTATCGGTTTGGTTGATCTGCATCC
TACCAGCCGTCTTCTACGACTCC*CACTCTACA*AGGTGTTTGGGCGGGGG
TCCTAAAAGTGCGAAAAAGTGCTTAAG*TGCCGGTGTGTTTTTGTGTG*TT
TAGGTGAAGTCGCAAACTCTCCCCGTG*A**TAGTCTTGCCCTGGTTTCTTT
GTCTCACACTC*C

Figure 13. Sequence from cloning of TAIL-PCR product of pfus3. Sequence provide by the Snell lab.

To compare this sequence against all available genomes, a general nucleotide BLAST was performed. BLAST results against all genomes supplied by (NCBI) revealed that the sequence did match that of mitochondrial DNA (Fig. 15). The highest percentage match found yielded 188/201 identities, or a 93% match as shown in Figure 15.

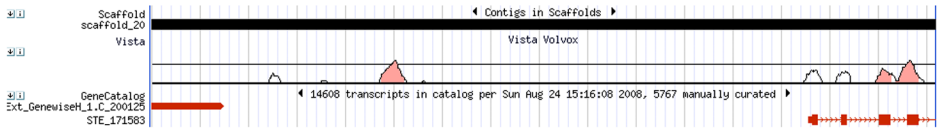


Figure 14. Sequence alignment for pfus3 TAIL-PCR sequence against the *Chlamydomonas* genome.

(A) pfus3 sequence alignment

```
264   CATGCCTAAGTGCGCAAACAGTATCGGTTTGGTTGATCTGCATCCTACCAGCCGTCTTCT  323
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
7     CATGCCTAAGTGCGCAAACAGTATCGGTTTGGTTGATCTGCATCCTACCAGCCGTCTTCT  66

324   ACGACTCCNCACTCTAC-ANAGGTGTTTGGGCGGGGGTCCTAAAAGTGCGAAAAAGTGCT  382
      |||||||   |||   | | | ||||||||||||||||||||||||||||||||||||
67    ACGACTC-GCACGATCCTATAGGTGTTTGGGCGGGGGTCCTAAAAGTGCGAAAAAGTGCT  125

383   TAAGNTGCCGGTGTGTTTTTGTGTGNTTTAGGTGAAGTCGCAAACTCTCCCCGTGNANNT  442
      ||||  |||||||||||||||||||  |||||||||||||||||||||||||||  |   |
126   TAAGGTGCCGGTGTGTTTTTGTGTGTTTTAGGTGAAGTCGCAAACTCTCCCCGTGTAGCT  185

443   AGTCTTGCCCTGGTTTCTTTG  463
      |||||||||||||||| |||||
186   AGTCTTGCCCTGGTTACTTTG  206
```

(B) pfus3 sequence alignment score

Score = 304 bits (336), expect = 3e-79
Identities = 118/201 (93%), Gaps = 2/201 (0%)
Strand=Plus/Plus

Figure 15. (A) Sequence alignment for pfus3 TAIL-PCR sequence against *Chlamydomonas* mitochondrial DNA. (B) pfus3 - mitochondrial DNA sequence alignment score.

III. pfus4 Sequence Analysis

Genomic DNA from pfus4 was isolated and a TAILPCR performed the Snell lab yielding the resultant band shown in Figure 16. This band was isolated, ligated into the

cloning vector, and sequenced at UT-Southwestern (Fig. 17). Using the provided sequence, I performed a nucleotide sequence BLAST analysis shown in Figure 18.

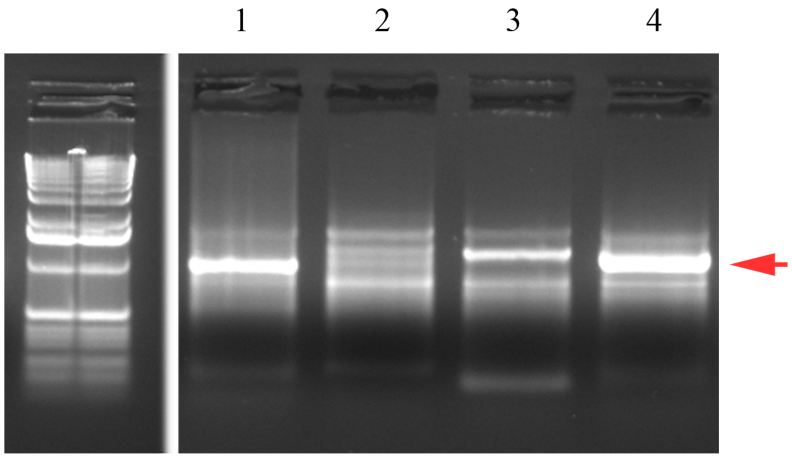


Figure 16. Tail PCR using 1 degenerate primer and three nested primers using fus4 genomic DNA was performed. Lane 1 = first amplification using degenerate primer and nested primer 1. Lane 2 = PCR reaction using degenerate primer and nested primer 2 with PRC 1 product as template. Lane 3 = third amplification using degenerate and nested primer 3 using second PCR product as template . Lane 4 = final amplification using degenerate and nested (4) primers using PCR product from third PCR reaction as template. Arrow indicates band in lane 4 that was ligated into the cloning vector and sequenced. Gel image provided by the Snell lab.

The BLAST analysis of the DNA flanking the insert in pfus4 against the *Chlamydomonas* genome showed highest percentage match found yielded 188/201 identities, or a 93% match to the ida5 Gene (Fig. 18). After running a BLASTX, using the translated nucleotide sequence, it was determined that the interrupted gene coded for actin. This gene was first identified and described by Sugase, *et al*, in 1996.

pfus4

CCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGTG
TGGACGTTGGGATCCCCGCTCCGTGTAAATGGAGGCGCTCGTTGATCTGAG
CCTTGCCCCCTGACGAACGGCGATGGATGGAAGATACTGCTCTCAAGTGCT
GAAGCGGTAGCTTGGCTCCCCGTTTCGTGCTGATCATTCCAGGTTTCAATGG
GTGGGCTAGGTGGGCCGGTTGGTTTTGTTTGGGGGGGGTTGCAGTGAGTG
GATAGTGGGTTGGATGCAGAACGGCATGATAGGACAGGACAGCATGGTGCA
TGTGTCAAGCAGGAGCCTCAGGCCAGCCAGCTGTCAGACAAGGCCAAGGT
GGCGACTTGCAGTCTGCATCACATGCGGGAGGGTGGGGGAGTCGATATGGG
CAAGCGGAGTCGGAGGTTAGGTCACGATGGCAGCGAACCGCCGACGGGCA
GCATGGCGCGGCATTGCCTTGCGCCGGAGTCTGAAGCTAATCGCGTCACTG
CTCGTGAGAAGCAGATTCTAATCAATGCCAGGGTGGTGAATGGCGTGAAGG
AAGGAACGAGGGAGTGGGGCAAGGCGTTGATTGGGGAAGCGATGCCCATA
TGGGGACCGGGAGGTCGTGGAACGTGGGGTGGGTGGTGTTGGAACGCAGT
CAGCAGTCAGTTGGGGAGGAAGGCAGCACAGTCACAATGGCCAACCGCCA
GCAGGATGTGCGCGAGCGGCATGCTGGACCCCAAACCGCCGCGGTCATGC
TTACATTTCCCCACCGAGCTAACCACAACATCTCCTTCCTCCTCCCTTCGAC
AGAGCGCGGCTACTCGTTCACCACCAC*GCCGAGCGCGAAATCGTGCGCA*
*ATCAAGGAGAAGCTGTGCTACGTGG*CC*GG*CTTCGAGCAGGAGATGG

Figure 17. Sequence from cloning of TAIL-PCR product of pfus4.

Discussion

The ultimate objective of this study was to identify at what stage or stages of the fertilization process and their associated genes were disrupted in three *Chlamydomonas reinhardtii* mating type plus mutants. These mutants were initially identified phenotypically based on their failure to form zygotes when mixed with mating type plus gametes based on bulk sample analysis. We found this lack of zygote formation was consisted throughout this study. All three mutants showed somewhat similar phenotypes when specific steps of the fertilization process were examined. Failure to form the fertilization tubule was consistent in all three mutants. Phenotypic characterization ruled out a number of steps that were unhindered and provided a base from which to begin the genetic analysis. Genetic analysis of these mutants resulted in mixed results.

(A) pfus4 sequence alignment

```
190   TTCCAGGTTTCAATGGGTGGGCTAGGTGGGCCGGTTGGTTTTGTTTgggggggTTGCAG  249
      ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
2216  TTCCAGGTTTCAATGGGTGGGCTGGGTGGGCCGGTTGGTTTTGTTTGGGGGGGGTTGCAG  2275

250   TGAGTGGATAGTGGGTTGGATGCAGAACGGCATGATAGGACAGGACAGCATGGTGCATGT  309
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2276  TGAGTGGATAGTGGGTTGGATGCAGAACGGCATGATAGGACAGGACAGCATGGTGCATGT  2335

310   GTCAAGCAGGAGCCTCAGGCCAGCCAGCTGTCAGACAAGGCCAAGGTGGCGACTTGCAGT  369
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2336  GTCAAGCAGGAGCCTCAGGCCAGCCAGCTGTCAGACAAGGCCAAGGTGGCGACTTGCAGT  2395

370   CTGCATCACATGCGGGAGGGTGGGGGAGTCGATATGGGCAAGCGGAGTCGGAGGTTAGGT  429
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2396  CTGCATCACATGCGGGAGGGTGGGGGAGTCGATATGGGCAAGCGGAGTCGGAGGTTAGGT  2455

430   CACGATGGCAGCGAACCGCCGACGGGCAGCATGGCGCGGCATTGCCTTGCGCCGGAGTCT  489
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2456  CACGATGGCAGCGAACCGCCGACGGGCAGCATGGCGCGGCATTGCCTTGCGCCGGAGTCT  2515

490   GAAGCTAATCGCGTCACTGCTCGTGAGAAGCAGATTCTAATCAATGCCAGGGTGGTGAAT  549
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2516  GAAGCTAATCGCGTCACTGCTCGTGAGAAGCAGATTCTAATCAATGCCAGGGTGGTGAAT  2575

550   GGCGTGAAGGAAGGAACGAGGGAGTGGGGCAAGGCGTTGATTGGGGAAGCGATGCCCATA  609
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2576  GGCGTGAAGGAAGGAACGAGGGAGTGGGGCAAGGCGTTGATTGGGGAAGCGATGCCCATA  2635

610   TGGGGACCGGGAGGTCGTGGAACGTGGGGTGGGTGGTGTTGGAACGCAGTCAGCAGTCAG  669
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2636  TGGGGACCGGGAGGTCGTGGAACGTGGGGTGGGTGGTGTTGGAACGCAGTCAGCAGTCAG  2695

670   TTGGGGAGGAAGGCAGCACAGTCACAATGGCCAACCGCCAGCAGGATGTGCGCGAGCGGC  729
      |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
2696  TTGGGGAGGAAGGCAGCACAGTCACAAT-GCCAACCGCCAGCAGGATGTGCGCGAGCGGC  2754

730   ATGCTGGACCCCAAACCGCCGCGGTCATGCTTACATTTCCCCACCGAGCTAACCACAACA  789
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2755  ATGCTGGACCCCAAACCGCCGCGGTCATGCTTACATTTCCCCACCGAGCTAACCACAACA  2814

790   TCTCCTTCCTCCTCCCTTCGACAGAGCGCGGCTACTCGTTCACCACCACNGCCGAGCGCG  849
      |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
2815  TCTCCTTCCTCCTCCCTTCGACAGAGCGCGGCTACTCGTTCACCACCACGGCCGAGCGCG  2874

850   AAATCGTGCGCANNATCAAGGAGAAGCTGTGCTACGTGGNCCNGGNCTTCGAGCAGGAGA  909
      ||||||||||   |||||||||||||||||||||||| || || ||||||||||||||||
2875  AAATCGTGCGCGACATCAAGGAGAAGCTGTGCTACGTGGCCCTGGACTTCGAGCAGGAGA  2934

910   TGG  912
      |||
2935  TGG  2937
```

(B) pfus4 sequence alignment score

Score = 1265 bits (1402), expect = 0.0
Identities = 714/723 (98%), Gaps = 1/723 (0%)
Strand=Plus/Plus

Figure 18. (A) Sequence alignment for pfus4 TAIL-PCR sequence against *Chlamydomonas* actin gene. (B) pfus4 - actin sequence alignment score.

Stages of Fertilization

Several events in the fertilization process of *Chlamydomonas* have been identified. The first stage is the formation of gametes from asexually reproducing vegetative cells. These haploid cells undergo gametogenesis when exposed to a number of external stimuli (Beck and Acker, 1992, Saito *et al.*, 1998, Treier *et al.*, 1989). The loss of nitrogen in the external environment is the most common method for experimentally inducing gametogenesis. Gametogenesis involves the expression of numerous gamete-specific genes (Treier *et al.*, 1989); included are genes for flagellar agglutinins in both mt+ and mt- gametes (Ferris and Goodenough, 2000), hap2 (Liu *et al.*, 2008) and mid in mt- (Ferris and Goodenough, 1997), *fus1* in mt+ (Ferris *et al.*, 1996). Gametogenesis typically occurs over a 12 h period and may or may not involve a cellular division.

Once gametes are formed there is a serves of cascade events leading to cell fusion and the formation of the zygote. When mt+ and mt- gametes are mixed they initially adhere via their flagella. Two mating type-specific flagellar agglutinin molecules bind with one another to cause this adhesion. This induces gamete activation – a series of events preparing the cells for fusion. Agglutination typically occurred with minutes of mixing gametes of the opposite mating type. Agglutinins are relatively short-lived, and new agglutinin molecules must be recruited from the cell body into the flagella (Hunnicutt *et al.*, 1990). Cell body agglutinins are inactive and become active upon recruitment to the flagella. If flagellar recruitment of agglutinins is prevented, the existing agglutinin is quickly lost and the gamete will detach from one another (Snell, 1981).

Aside from flagellar recruitment, adhering flagella also exhibit tipping (Goodenough, 1993, Goodenough and Jurivich, 1978). During this process agglutinin molecules are

preferentially recruited to the tips of the flagella leading to a distinct flagellar adhesion pattern (Goodenough, 1993).

Flagellar agglutination leads to a signaling cascade that induces a number of downstream gamete activation events. One event is the release of the enzyme lysin from periplasm stores (Buchanan *et al.*, 1989). Lysin causes the cell wall to degrade in spots allowing the cell body to escape the extracellular covering. This exposes the cell membrane allowing ultimate to fuse the appropriate gamete.

One known step in the signaling pathway from flagellar agglutination to cell wall loss is the production of intracellular cAMP by adenylate cyclase (Pan and Snell, 2000). The steps upstream of cAMP elevation are unknown as are the specific steps downstream leading to lysin release. Artificially raising intracellular cAMP levels by bathing cell in membrane permeable dibutryl cAMP will induce downstream cell wall loss (Pasquale and Goodenough, 1987).

Another significant event in the flagellar agglutinative inducing gamete activation pathway is the activation of the mating structures. In plus gametes, this involves the polymerization of actin filaments to form the fertilization tubule. Elevation of intracellular cAMP is essential for fertilization tubule formation and artificial elevation of cAMP by incubation in the membrane permeable dibutryl cAMP will induce fertilization tubule formation (Wilson *et al.*, 1997). A *Chlamydomonas* mutant defective in the actin gene (ida5) failed to form fertilization tubules (Kato-Minoura *et al.*, 1997). Moreover, these mutants showed low levels of successful fertilization presumably due to the importance of an extended fertilization tubule.

Along with the structural formation of the fertilization tubules is the recruitment of extracellular fus1 protein along the elongated fertilization tubule (Misamore *et al.*, 2003). In the unactivated mating structure, fus1 exists as a distinct patch on the surface of the mating structure. With the formation of the elongated fertilization tubule, Fus1 is distributed along the length of the fertilization tubule (Misamore *et al.*, 2003). Fus1 is essential for adhesion of the fertilization tubule to the minus mating structure. A *Chlamydomonas* mutant defective in the fus1 gene (fus1-1) forms a fertilization tubule structurally similar to wt+ fertilization tubules. However, the absence of the fus1 protein prevents the adhesion of fus1-1 fertilization tubules to wt- mating structures. Fus1-1 mutants show minimal to no ability to undergo fertilization (Kato-Minoura *et al.*, 1997).

Pfus2

Phenotypic analysis of pfus2 gametes showed several functioning steps in the fertilization process. These cells were able to undergo gametogenesis at least to some degree. Depriving them of nitrogen overnight resulted in cells that produced plus agglutinins. Production of these proteins is limited to gametes (Bergman *et al.*, 1975). Moreover, the pfus2 gametes also produced lysin, another gamete specific protein (Goodenough and Weiss, 1975). These results suggest that genes regulating the gametogenic process appear to function normally.

Pfus2 gametes adhered to wt- gametes by flagellar agglutinins in a pattern similar to wt+ gametes. Both wt+ and pfus2 gametes exhibited high levels of agglutination within 10 minutes of mixing with wt mt- gametes. *Chlamydomonas* typically occurs within minutes of mixing gametes of the opposite mating type.

Agglutination continued for at least 30 min after mixing. The presence of continued agglutination between pfus2 and wt+ gametes suggests two conclusions. First, pfus2 gametes are able to recruit new agglutinin molecules to their flagella to sustain continued adherence to wt- flagella. Second, pfus2 gametes are able to generate significant signaling within wt- gametes to induce the continued recruitment of minus agglutinins to the wt- flagella to maintain continued agglutination. These two finding suggest that flagellar agglutination and subsequent signaling for agglutinin recruit is not altered by the insertional mutation.

The release of lysin inducing cell wall loss in pfus2 gametes showed a similar pattern to that of wt+ gametes. Pfus2 gametes showed a large degree of cell wall loss when mixed with wt- gametes by 30 minutes. Thus, sufficient quantities of enzymatically active are released by pfus1 gametes to allow for cell wall loss.

The signaling pathway from flagellar agglutination leading to gamete activation to the release of lysin and the loss of the cell wall appears to function properly. Moreover, pfus2 were able to respond to artificial activation by dbcAMP leading to the release of lysin and cell wall loss. Thus, the signaling pathway from flagellar agglutinin through cAMP elevation leading to lysin release appears to be functioning normally in pfus2 mutants.

Gamete activation induced by flagellar agglutination with wt- gametes did not produce fertilization tubule formation in Pfus2 gametes as seen in wt+ gametes. This suggests either a component of the signaling pathway leading to fertilization tubule formation is disrupted or a structural component essential for fertilization tubule formation is absent. The only know step in the fertilization tubule formation signaling pathway is elevation of cAMP. The disruption of this pathway may occur either upstream, at cAMP production, or

downstream of cAMP elevation. Bypassing upstream and production events by incubation of pfus2 gametes with dbcAMP failed to induce fertilization tubule formation. This suggests that if there is a disruption in the pathway, it should be located downstream of cAMP elevation.

The only known structural protein present in fertilization tubules is actin. We observed no detectable actin in the pfus2 mutants. However, a distinctive characteristic of actin-defective, ida5 mutants is a greatly reduced ability to swim. We did not observe this behavior in pfus2. However, direct screening the intact actin gene is required to determine whether pfus2 is not another actin-defective mutant. Additionally, several other structural genes known to associate microvilli in other species are expressed in Chlamydomonas (Szopinski, 2004). While gene transcription occurs, the associated protein and their possible role in fertilization tubule formation are unknown. However, these genes are also possible candidates along with actin for the pfus2 mutation.

Since fertilization tubules in pfus2 failed to form, the proper function of fertilization events downstream of fertilization tubule formation can not be investigated. Any steps beyond fertilization tubule formation require the presence of the fertilization tubule in the mt+ gamete. Although the absolute requirement of an elongated fertilization tubule for successful fertilization is somewhat debated, rates of zygote formation are minimal at best (Kato-Minoura *et al.*, 1997). Without forming a viable fertilization tubule, the pfus2 gametes did not show gametic fusion and did not form quadriflagellate zygotes as wt mt+ gametes do when mixed with wt mt- gametes.

Disruption of the fus1 gene in pfus2 leading to infertility seems unlikely. While we did not screen directly for an intact fus1 gene, several indirect pieces of evidence suggest that

pfus2 is not another fus1 mutant. First, all three know fus1 mutants (fus1-1,1-2, 1-3) form normal fertilization tubules while pfus2 does not. Second, cursory staining of pfus2 gametes with the fus1 antibody showed detectable fus1 protein (M. Misamore, personal communication); however, the unstable nature of currently available fus1 antibody requires conservative conclusions regarding the absence of fus1 protein. It is worth noting that fus1 protein is lost in the actin mutant ida5 even though the fus1 gene is intact (Misamore, personal communication).

Genetic analysis of the pfus2 mutant has proven to be highly problematic. TAIL-PCR appeared to produce one promising band that could lead to flanking DNA and ultimately the disrupted gene. Repeated attempts to directly sequence the PCR product proved unsuccessful. Moreover, repeated attempts to ligate the PCR product into a cloning vector have been unsuccessful. Further attempts at producing a new TAIL-PCR product have also yielded no new results. In order to fully define this mutant, determination of alternate methods that produce a positive TAIL-PCR and cloning of the fragment DNA is needed.

Pfus3

Pfus3 gametes showed many similarities in phenotype, to wt+ gametes. When deprived of nitrogen, pfus3 vegetative cells underwent gametogenesis. This was verified by the production of two gamete specific proteins, plus agglutinins and lysin (Bergman *et al.*, 1975, Goodenough and Weiss, 1975). Thus, the general signaling pathway inducing gametogenesis appears to be functioning in pfus3 mutants

Pfus3 gametes produced plus flagellar agglutinins. When mixed with wt- gametes, their flagella bound to minus agglutinins on the wt- flagella. The majority of the cells were

fully agglutinated within 10 minutes of mixing with wt mt- gametes. Pfus3 mutants also appear to recruit new agglutinin molecules to the flagella. Agglutination continued for over 30 min while agglutination quickly terminated when agglutinin recruitment was prevented (Hunnicutt and Snell, 1991). There was a slight difference in agglutination pattern in pfus3 relative to wt+ gametes. Pfus3 gametes showed a reduction in size and number of agglutinating clumps between 10 minutes and 15 minutes relative to wt+ gametes. Smaller, more dispersed clumps formed. While this suggests a possible effect of the pfus3 mutation on agglutination, development of more precise, quantitative methods for measuring agglutinative are needed to verify this phenotype. Nevertheless, pfus3 mutants exhibited agglutination and flagellar agglutinin recruitment. This suggests that the disrupted gene in the mutant is not related to expression of agglutinins or any other genes involved in agglutination that may, as yet, be undiscovered.

When activated by flagellar agglutination, pfus3 gametes released lysin and released their cell walls at a rate equal to that of wt mt+ gametes. The pfus3 gametes showed a high degree of cell wall loss by 15 minutes and almost all cells had shed their walls by 30 minutes. Similar rates were observed in wt+ gametes. Pfus3 gametes also responded to artificial activation by dbcAMP by releasing lysin and losing their cell walls. Cell wall loss, induced by either flagellar agglutination or cAMP elevation, suggests that the signaling pathways and mechanisms involved in cell wall loss are functioning correctly in pfus3.

Pfus3 gametes produced no fertilization tubules when gamete activation was induced either via mixing with wt mt- gametes or induction with dbcAMP. As with pfus2, there was no detectable actin polymerization associated with pfus3 mutants. This suggests a disruption in either the assembly of the fertilization tubule or the signaling pathway leading to

fertilization tubule formation. If the pfus3 mutation is in the signaling pathway, the disruption must be downstream of cAMP elevation. Possible structural genes that would be disrupted in the pfus3 mutant include actin, fimbrin, villin, or myosin I. Again, the absence of a viable fertilization tubule leaves the pfus3 gametes incapable of gametic fusion and thus, the gametes did not form a quadriflagellated zygote when mixed with wt mt- gametes.

As described for pfus2, the possibility that pfus3 is another fus1 mutant exists. This seems unlikely for the same reasons given for pfus2. Pfus3 failed to form a fertilization tubule while all previous fus1 mutants exhibited normal tubule formation (Ferris *et al.*, 1996). Additionally, initial labeling the fus1 antibody showed no obvious presence of the fus1 protein on pfus3 gametes.

Initial analysis of the pfus3 TAIL-PCR sequence against the *Chlamydomonas* genome revealed a sequence that fell between genes. The implications of a sequence falling between predicted gene sequences could possibly be that the insert has fallen on a gene promoter upstream of one of the flanking genes. It is also possible that the insert may have broken up an enhancer that affects the transcription of any number of genes at multiple loci throughout the genome. The complexity of this situation makes pfus3 a less desirable mutant to work with when compared to the other mutants.

When the sequence was run against all genomes the sequence was found to be a piece of mitochondrial DNA. Mitochondrial DNA is removed from the *Chlamydomonas* genome database and was therefore missed in the original analysis of the sequence. However, most mitochondrial DNA mutations are either lethal or require specific growth conditions for survival (Matagne et al., 1989).

This result does not mean that the TAIL-PCR provided an inaccurate sequence, rather that the mutation may be more complex than a simple interruption of a gene directly involved in fertilization. The mutation could possibly be a mitochondrial defect, effecting the formation of FT. Furthermore, and just as likely, the insert could have landed in the promoter region of a gene downstream of a gene that is directly involved in fertilization. The sequence did fall upstream of a gene coding for SNARE proteins, a possible gene of interest considering that SNARE proteins are important in vesicle formation (Feng *et al,* 2009), a process that may have similarities to fertilization tubule elongation (Wilson, 1996).

Pfus4

Pfus4 gametes were no different than wt mt+ gametes in all stages of fertilization leading up to fertilization tubule formation. As was the case with pfus2 and pfus3 gametes, pfus4 gametes showed full agglutination within 10 minutes of mixing with wt mt- gametes. Pfus4 mutant gametes showed a high degree of cell wall loss by 15 minutes and nearly all cells showed cell wall loss at the 30 minutes time point. Induction of cell wall loss by addition of dbcAMP also yielded complete CWL, as was the case with wt mt+ gametes.

Pfus4 gametes yielded no fertilization tubule formation regardless of whether gamete activation was induced by mixing with wt mt- gametes or induction with dbcAMP. As described earlier, in the absence of the fertilization tubule the pfus4 gametes are incapable of gametic fusion and therefore cannot form a zygote when mixed with wt mt- gametes.

The BLASTN and subsequent BLASTX analysis of the pfus4 TAIL-PCR sequence revealed that the sequence was that of mRNA coding for Chlamydomonas actin. Actin, as stated earlier, has been found to play a role in elongation of the FT. The polymerization of Actin, just below the plasma membrane, is necessary for the formation of the FT. The

function of this actin gene in Chlamydomonas was first reported by (Kato-Minoura *et al.*, 1997). The phenotype of the pfus4 mutant is similar to the actin-defective, ida5 mutant. Both pfus4 and ida5 fail to exhibit any appreciable polymerized actin upon gamete activation. Neither forms an elongated fertilization tubule. Given the pfus4 mutant had the same phenotype and genotype as the *ida5* mutant, it was decided that the pfus4 mutant was simply an *ida5* mutant.

Future Study into pfus2 and pfus3

Further study is needed in both pfus2 and pfus3 to determine whether or not these two mutants are of particular interest to move forward, or if these mutants are similar to other previously defined mutants. Further study begins, with successful sequencing of the DNA flanking the insert, in pfus2, and additional attempts at TAIL-PCR sequencing for pfus3.

Successful sequencing of the TAIL-PCR product of pfus2 will allow, first and foremost, whether or not to continue forward. Secondly, the gene discovered to be interrupted will have to be studied for its protein products. RT-PCR to verify that the gene is not being translated and subsequently transcribed can verify the gene's interruption. A subsequent rescue study which should definitively verify the interruption of the gene of interest and define its role in fertilization.

The case of pfus3 is a bit more complex at this point. It is unknown as to whether the TAIL-PCR sequence provided was correct, or if additional sequencing attempts will yield significantly different results. Even with verification of an accurate sequence, the problem remains an intricate one. To rule out or confirm the interruption of an upstream promoter, RT-PCR for the product of a gene of interest should provide definitive results. A positive

result showing that the mutant is not transcribing the gene of interest would produce an experimental pathway similar to that described for pfus2.

A negative result would lead in another direction. With multiple sequences showing that the insert truly is between areas of genomic DNA, and results proving that the interrupted area was not a promoter for a gene involved in fertilization, it could be determined that the interrupted sequence was that of mitochondrial DNA. This would lead to a new study into the role of mitochondria in fertilization tubule formation, and fertilization as a whole.

Summary

These three mutants have yielded very interesting results. While the pfus4 mutants has been classified as an ida5 mutant, the other two mutants, pfus2 and pfus3, are still mutants of interest for finding a gene directly tied to fertilization tubule formation. The genes interrupted in pfus2 and in pfus3 are certainly, directly involved in fertilization tubule formation, as both mutants do not form fertilization tubules, but it is unclear as to how those genes are involved. There are various paths that could be taken to determine the identity of the interrupted gene.

For pfus2 a TAIL-PCR sequence falling in an area of genomic DNA is necessary to continue forward. The low yield process of TAIL-PCR is, at the moment, the only method that could find the area flanking the insert. After a positive TAIL-PCR sequencing, knockout and rescue experiments should verify whether or not the sequence returned was that of the interrupted gene.

The situation for pfus3 is a bit more complicated, as a TAIL-PCR sequence has already come back positive for *Chlamydomonas* DNA. However, the sequence fell between

genes coding for genomic DNA when run against the *Chlamydomonas* genome, and was identified as *Chlamydomonas* mitochondrial DNA when run against all mapped genomes. This could mean a number of things. The insertion may have fallen in a promoter region upstream of a gene involved in fertilization tubule formation. However, the sequence alignment could be correct and fertilization tubule formation could be controlled in some manner by mitochondrial DNA. It is certain though that another positive TAIL-PCR sequence is necessary to provide a little more certainty that the first sequence is the correct sequence for the area flanking the insert. Again, knockout and rescue experiments will prove or disprove that this gene is directly involved in fertilization tubule formation.

The fertilization tubule is extremely important in the fertilization process. These mutants could provide much needed information in mapping out the genetic process of fertilization. Finding the gene, or genes, involved in creating fertilization tubules could provide a method for improving or decreasing fertility at will. These mutants are not yet classified genotypically and could still hold important information that would lead to a far greater understanding of the fertilization process as a whole.

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VITA

Condred Curtis Roberts III was born in Fort Polk, LA on November 16, 1983. He moved permanently to Houston, TX with his parents and brother in 1995. He is the son of Condred Curtis Roberts Jr. and Marilyn Jo Roberts. He has one brother, Gregory Roberts. Condred graduated from Bellaire High School in 2002.

After high school Condred went to Texas Tech where he graduated in 2007 with a B.S. in Zoology and a minor in Chemistry. Condred moved directly into his graduate work at TCU where he studied Biology under the guidance of Dr. Michael Misamore.

Condred is currently employed by the University of Texas Southwestern Medical Center where he is a Research Associate in the Department of Pharmacology.

ABSTRACT

Characterization of three sexually sterile mutants of the green alga *Chlamydomonas reinhardtii*

By Condred Roberts, M.S., 2009

Department of Biology

Texas Christian University

Thesis Advisor: Dr. Michael Misamore, Assistant Professor of Biology

The focus of this study was to identify the phenotype and genotype of three mutants of the green alga *Chlamydomonas reinhardtii*. These three mutants, all of the mating type plus, were created by random insertional mutagenesis, and subsequently screened for their inability to form zygotes when mixed with mating type minus gametes. To characterize individual phenotypes, the mutants were observed at each individual stage of the fertilization process, from agglutination through mating structure fusion and zygote formation. All three mutants showed normal progression of fertilization but failed to form fertilization tubules. Following phenotypic characterization, each mutant was subject to TAIL-PCR and DNA sequencing to determine the location of the insert. The location of the insert was determined in two of the three mutants. One mutant was defective in the actin-encoding gene (*ida*5) while the second was a possible insertion into a portion of mitochondrial DNA.