CLONING A GAMETE FUSION GENE IN *CHLAMYDOMONAS REINHARDTII*

by

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ABSTRACT

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Many biological processes require cell fusion, therefore defects in cell fusion result in many diseases. The unicellular green alga *Chlamydomonas reinhardtii* is an excellent model organism to study cell-cell fusion. Objective of this study was to identify genes that are involved in *Chlamydomonas* mating type minus gamete fusion. A forward genetics approach was taken in our work. We created several mating type minus fusion defective mutants using DNA insertional mutagenesis. These mutants were normal in early stages of mating; they agglutinated with mating type plus, removed their walls, adhered to their mating partner through mating structure, but cells did not fuse indicating that the DNA insertional mutants were defective in the latest stages of fusion. The number of insertions was confirmed by Southern blots. Mutant J1 had one insertion and the flanking genomic DNA was cloned by TAIL-PCR and RESDA-PCR. We complemented the mutant J1 with a BAC that contained two genes; we were able to get zygotes when mated with wild type mating type plus cells. We proved that the progeny of these zygotes were products of meiosis. The BAC used for complementation had 2

genes, we need to determine which gene complemented the phenotype. We also identified a new mutant, with a phenotype that has never been reported in the literature. It agglutinates with itself and forms pellicles that look like zygote pellicle.

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CHAPTER 1
INTRODUCTION

1.1 Fusion Review

Cell-cell fusion is involved in many biological processes; fertilization, formation of multinucleated muscle syncytia, development of bone and placenta, the immune response, tumorigenesis, and stem cell mediated tissue regeneration (Chen and Olson, 2005). In spite of the diversity of cell types that undergo fusion, the process of cell fusion, including cell-cell adhesion, alignment and membrane mixing are common in every cell type. Present data suggests that fusion reactions share common features, but are catalyzed by diverse proteins. These proteins mediate the initial recognition of the membranes that are destined for fusion and pull the membranes close together to destabilize the lipid/water interface and to initiate mixing of the lipids. A single fusion protein may do everything or assemblies of protein complexes may be required for intracellular fusion reactions to guarantee rigorous regulation in space and time (Jahn et al., 2003). Despite its importance, little is known about the mechanism of this process: How do cells recognize and fuse with each other? What are the specific membrane proteins and intracellular regulatory proteins involved in fusion? Does cell-cell fusion employ the same mechanism as other fusion events, such as intracellular vesicle fusion and virus-cell fusion? These are some of the questions remaining to be solved.

Proteins involved in virus-cell fusion and the intracellular vesicle fusion are the most well studied fusogens. To understand the mechanism of cell-cell fusion, it is helpful to look at these proteins and their mechanism.

1.1.1 Virus-Cell Fusion and Intracellular Vesicle Fusion

In enveloped viruses, the nucleocapsid with the genetic material is covered by a host-derived lipid bilayer. Enveloped viruses use transmembrane viral proteins that mediate fusion with the host cell membrane. Class I viral fusion proteins, such as influenza hemagglutinin (HA) and human immunodeficiency virus type 1 (HIV-1) envelope protein (Env), contain a hydrophobic fusion peptide that is normally buried within the molecule. The structure of HA at membrane pH (Bullough et al., 1994) and different pH conditions has been determined. The structure of the HIV Env glycoprotein has also been determined (Kwong et al., 1998; Wyatt et al., 1998). Before the virus buds from its parental host cells, the fusion proteins are processed by a proteolytic cleavage, resulting in an N-terminal globular and a metastable C-terminal part. The C-terminal part usually contains an amphiphilic fusion peptide of 15–30 amino acids that is adjacent to the cleavage site, and a transmembrane domain that anchors it on the virus. Upon activation of the fusion protein by a shift in pH (as in HA) or binding to a surface receptor (as in Env), the previously hidden fusion peptide is exposed and inserted into the target membrane. After insertion in the target membrane, the fusion protein undergoes a conformational change in which two separated α-helices fold upon each other to form a hairpin-like α-helical bundle, thereby bringing the viral and cell membranes into close proximity and resulting in membrane fusion (Jahn et al., 2003; Skehel and Wiley, 2000). Insertion of class II viral fusion proteins into the target membrane may be mediated by β barrels rather than amphiphilic α helices (Kuhn et al., 2002).

N-ethyl-maleimide-sensitive factor attachment protein receptors (SNAREs) which are involved in intracellular fusion events, also rely on α helical bundles. Vesicle anchored

(v-SNARE) and target anchored (t-SNARE) proteins interact to form a bundle of α-helices bringing membranes together and promote fusion (Jahn and Scheller, 2006). It is not clear if the SNAREs themselves have fusogen activity.

1.1.2 Yeast Fusion

S. cerevisiae has two mating types (sexes), *MAT**a*** and *MAT*α. MATα cells release the α-factor pheromone (Sag1), which binds to a G-protein coupled receptor on *MAT**a*** cells. Sag1 has three Ig like domains. The a-agglutinin binds to a G-protein coupled receptor on *MAT*α cells (Zhao et al., 2001b). The pheromone receptors activate a common MAP kinase signaling pathway resulting in fusion (Bardwell, 2005). Despite extensive genetic screens for fusion defective mutants in yeast, no mutants have been found which have a defect in membrane fusion, perhaps because of functional redundancy. All the mutants identified are defective in the early stages of mating, i.e before the cell wall lysis. However, through bioinformatic screenings it was possible to identify a protein that is involved in late stages of mating; pheromone regulated membrane protein (Prm1p). It is not clear if the Prm1p is a fusogen, because only 50% of the *Prm* mutants fail to fuse, furthermore fusion defect is only seen when Prm1p is deleted in both mating partners (Heiman and Walter, 2000). Prms have transmembrane domains but lack hydrophobic fusion peptides; therefore if they do cause fusion, their mechanism of action must be distinct from SNAREs and class I viral fusion peptides. The mechanism of plasma membrane fusion remains largely unknown. It is not known if the Prms interact with other proteins which may be fusogens. It may be possible to identify other proteins by looking at what is interacting with Prms (Chen et al., 2007).

1.1.3 Fusion in *C. elegans*

In *C. elegans*, one third of its somatic cells fuse to form a multinucleated syncytium. Fusion occurs throughout development and it is essential for formation of multiple organs. Genetic screens for fusion defective mutants have identified a gene named epithelial fusion failure 1 (*eff*-1) (Mohler et al., 2002). Ectopic expression of EFF-1 in normally non-fusing *C. elegans* cells (Shemer et al., 2004) and in Sf9 insect cells (Podbilewicz et al., 2006) promotes cell fusion. EFF-1 contains an extracellular hydrophobic domain and this domain is involved in localization of EFF-1 to the membrane but not in fusion-pore formation (Kontani et al. 2005).

AFF-1 (anchor fusion failure 1) mediates fusion of the anchor cells. AFF-1 ectopic expression results in fusion of cells that normally do not fuse in *C. elegans* (Sapir et al., 2007). Therefore both EFF-1 and AFF-1 are considered as fusogens.

Three sperm expressed proteins are known to be required for sperm fusion in *C. elegans*. Spe-9 is a single pass transmembrane protein with EGF motif (protein-protein interaction motif) (Putiri et al., 2004; Singson et al., 1998). Spe-38 is a tetraspanin but is not a homologue of mammalian CD9 (Chatterjee et al., 2005). Spe-41 is a calcium channel that is involved in sperm-egg interaction (Xu and Sternberg, 2003).

On the egg side, Egg-1 is known to be required for fusion and it localizes to egg surface. Egg-1 is an LDL receptor family protein. Mutants of *egg-1* fail to internalize Egg-1 (Kadandale et al., 2005).

1.1.4 Myoblast Fusion in Drosophila

Mononucleated myoblasts (muscle founder cells and fusion competent cells) fuse to form multinucleated muscle fibers. Genetic screens have identified many proteins that are involved in myoblast fusion. Ig- domain-containing cell surface receptors are found in the first class. These receptors, specifically expressed in fusion-competent or founder cells, mediate fusion. Intracellular proteins are found in the second class. It is unclear if these intracellular proteins are only involved in cytoskeletal remodeling or also in membrane destabilization to promote fusion (Chen et al., 2004).

1.1.5 Macrophage Fusion

Macrophages are present in every tissue and can fuse with other macrophages to differentiate into multinucleate osteoclasts (in bone) and giant cells (in many tissues) which play a central role in osteoporosis and immune response, respectively (Vignery, 2005). Bone is continuously broken down (resorbed) by osteoclasts and formed by another type of cells, the osteoblasts. Osteoporosis occurs when the rate of bone resorption surpasses that of bone formation. The bone degrading capacity of osteoclasts depends upon their physical interaction with receptor proteins on the bone surface. Macrophages might also fuse with somatic cells to promote tissue repair, and with tumor cells with to trigger metastasis. Putative fusion machinery that mediates macrophage fusion was identified using monoclonal antibodies that recognize and prevent fusion. The first protein identified is macrophage fusion receptor (MFR), an immunoglobulin (Ig) domain containing protein (Saginario et al., 1995). MFR binds to CD47, which is also in

Ig superfamily. Osteoclast formation is reduced in the absence of MFR/CD47 interaction (Lundberg et al., 2007).

1.1.6 Mammalian Fertilization

To date, genetic screens have identified an Ig domain containing transmembrane protein called Izumo on sperm (Inoue et al., 2005) and a tetraspanin found in the egg plasma membrane called CD9 (Primakoff et al., 2002) to be required for fusion. The specific role of these proteins in fusion promotion is not known. The role of transmembrane domains in fusion is still under investigation (Langosch et al., 2007).

1.1.7 Fusion of Placenta

In the mammalian placenta, trophoblasts fuse to form syncytial layer of cells that function as a barrier between maternal and fetal blood vessels. A protein called Syncytin is the only protein identified to cause cell fusion in this system. Syncytin is found only in primates and it is nearly identical to the envelope protein of human endogenous retrovirus. Therefore Syncytin might be a captured retroviral protein (Mi et al., 2000).

1.1.8 Stem Cell Fusion

It has been shown that hematopoietic stem cells can differentiate into several types of cells, including cardiac myocytes, hepatocytes, Purkinje cells, and oligodendrocytes as result of cell fusion (Pomerantz and Blau, 2004; Wagers and Weissman, 2004). It is not known what proteins might be involved in these fusion events.

1.1.9 Diseases Involving Fusion and Importance of Fusion

Defects in fusion processes result in many diseases. Failure of sperm-egg fusion is a major cause of infertility. Certain muscle diseases also result from fusion defects. Defects in placental trophoblast fusion result in pregnancy complications. Defects in osteoclast fusion cause bone abnormalities such as osteoporosis.

Unregulated fusion also can cause diseases such as cancer. Many cancer cells are fusogenic. In most cases the fusion product is more malignant then the original cell. Fusion may also produce diverse cancer types (Duelli and Lazebnik, 2003).

Fusion can be used as a tool to induce tissue regeneration. It is therefore important to identify proteins involved in fusion events for therapeutic purposes. Study of fusion proteins identified to date suggests that, cell-cell fusion is not likely to be mediated by common molecules in every system; it is rather the act of different molecules in different cell types.

1.2 Life Cycle of *Chlamydomonas*

Chlamydomonas reinhardtii is a unicellular, biflagellated, eukaryotic green alga. It is approximately 10μm long. Most of its life cycle is in the vegetative haploid stage. Vegetative cells divide by mitosis. When deprived of nitrogen, simply by placing cells in nitrogen free medium in the laboratory, they go through gametogenesis and become mating competent (Sager and Granick, 1954). The gametes demonstrate several differences from the vegetative cells they are derived from. Molecular control of gametogenesis is still under investigation and will be discussed below. Gametes fuse to form a diploid zygote, which can stay dormant for months when kept in the dark. When

the environmental conditions are normal and in the presence of light, a zygote undergoes meiosis and forms four or eight haploid progeny.

The mating reaction of *Chlamydomonas* is a useful system for understanding the nature of gamete fusion (fertilization). *Chlamydomonas* has two mating types (sexes): mating type plus (mt^+) and mating type minus (mt^-). The mating type is determined by the mating type locus, which is located in the left arm of linkage group (chromosome) VI. The genes that are found in this locus determine if the cells will mate as mt^- or mt^+. The mating type locus is under recombinational repression to assure that *mt*-linked genes are segregated together. The haploid progeny that receives either allele (mt^- or mt^+) maintains its type without any mating type switching. This suggests that the expression of these genes is dependent on one another (Ferris et al., 2002; Goodenough et al., 2007). When the gametes of opposite mating types are mixed together, they recognize each other with their flagella by glycoproteins called agglutinins found on the flagellar surface (Adair et. al., 1983). Plus agglutinin is encoded by *Sag1* and minus agglutinin is encoded by *Sad1*. *Sag1* is unlinked to mt-locus, but *Sad1* is located in the mt^- locus (Ferris et al., 2002; Ferris et al., 2005). *Sad1* is also found in mt^+ but not expressed, because its expression requires *mid* (minus dominance) which is only found in the mt^- locus (Ferris et al., 2002).

After recognition, agglutination occurs and the flagella undergo tipping, a preference for interaction at the tips (Bergman et al., 1975; Forest et al., 1978). Agglutination induces a change in the flagellar tips that appears to be essential for further events in mating to occur. The flagellar tip enlarges and fibrous material accumulates in a specific region between the nine singlet A microtubules and the terminal membrane (Mesland et al.,

1980). This leads to a series of signaling events. It was suggested that transmission of the sexual signal is mediated by calcium fluxes across the flagellar membrane (Snell et al., 1982). Flagella interactions initiate a protein-kinase dependent signaling pathway that leads to activation of a gamete specific adenylyl cyclase. This leads to a rapid increase in the intracellular cAMP concentration (Pasquale and Goodenough, 1987). An intraflagellar transport system delivers more agglutinins to the flagella surface from the cell body (Wang et al., 2006). Signaling results in secretion of a serine protease required to convert a periplasmic prometalloprotease to an active matrix degrading metalloprotease (autolysin) (Buchanan et al., 1989; Snell et al., 1989). This release leads to degradation of the cell wall (Matsuda et al., 1985). In the normal course of mating, cell wall lysis is one of the events triggered by flagellar tip activation in *Chlamydomonas reinhardtii* and can be recognized by the rapid appearance of carbohydrates in the culture medium (Solter and Gibor, 1977). Cell wall lysis can also occur during gametogenesis (Matsuda, 1980). Signaling also leads to activation of mt^+ mating structures by actin polymerization (Goodenough and Weise, 1975). The mating structures are located next to one of the flagella in both mating types. The activated mt^+ mating structure is a long microfilament filled, acrosome like extension (Goodenough and Weiss, 1975). At the tip of mt^+, there is a glycoprotein layer called fringe (Goodenough et al., 1982). Activation of the mt^- mating structure results in a slight elevation of the plasma membrane (a dome like structure) (Forest, 1983). Cells mobilize membrane particles to the center of the mating structure, which was cleared of particles during gametogenesis (Goodenough et al., 1982; Weiss et al., 1977). The tip of the mt^+ mating structure recognizes and adheres to the activated mt^- mating structure (Forest, 1983). The mt^+ structure "fringe" is made of

FUS protein that is involved in adhesion to the *mt*$^-$ structure (Ferris et al., 1996; Misamore et al., 2003). On the *mt*$^-$ structure, GCS1 (Generative Cell Specific 1) has been identified (Mori et al., 2006). GCS1 has been identified in lily and found to have a homologue in *Chlamydomonas.* It is a transmembrane protein that localizes to the *mt*$^-$ structure (Liu et al., 2008). Its expression is in low levels in vegetative cells of both mating type, but increased in *mt*$^-$ gametes. Once adhesion and fusion of the fertilization tubule and the *mt*$^-$ mating structure occur, the tubule rapidly shortens and brings the apical ends of the cells into opposition (Friedman et al., 1968). This adhesion results in gamete fusion and production of the diploid quadriflagellated cell (QFC), or zygote. After a few hours, the zygote resorbs its flagella and forms a thick zygospore wall. Following maturation in dark (for a minimum of 6 days), the zygote germinates upon returning to light, undergoes meiosis and forms four or eight haploid progeny. (See figure 32 for images of mating).

1.3 Transcriptional Program of Gametogenesis in *Chlamydomonas*

To determine which genes were upregulated under nitrogen deficient conditions, Abe et. al. performed an EST-based macroarray analysis (Abe et al., 2004). Briefly, they synchronized cells in +N and in –N medium by 12 hrs light, 12 hrs dark incubation and isolated mRNA every hour for 8 hrs. They made cDNA from these two pools of mRNA and during cDNA synthesis labeled with ^{32}P. This cDNA was then hybridized to an EST library. This study shows the temporal expression of genes that were upregulated under –N conditions. 18 new genes were identified some of which don't have any known function. However, no proteins with transmembrane domains were identified (we would

predict a fusion protein would have at least one).

1.4 Hypotheses and Specific Aim

Our goal is to identify the genes that are involved in mt^- gamete fusion in *Chlamydomonas.* All the fusion defective mt^+ mutants are known to be defective in mating structure adhesion, and mt^- fusion defective mutants are known to adhere but do not fuse (Forest, 1987). Our hypothesis is that there are one or more molecules on the mt^- mating structure that are required for fusion to occur. Our goal is to isolate fusion defective mt^- mutants that are defective in the late stages of mating and identify the genes that are being disrupted. Once we identify the disrupted genes, we will then use the wild type version of these genes for complementation of the mutants, thereby rescuing the wild type phenotype.

CHAPTER 2

MATERIALS AND METHODS

2.1 Creation and Selection of Fusion Defective Mutants

2.1.1 Proteolytic Enzyme (Autolysin) Preparation

The strains of *Chlamydomonas* used to make autolysin were CC-620 R3 MN mt^+ and CC-621 NO mt^-, because these strains have high mating efficiency. Cells were grown on four 150 x 15 mm petri dishes (2 plates of mt^+ and 2 plates of mt^-) for 12-14 days. To make the autolysin, plates were flooded with 20 ml SEM-N so the cells would grow flagella and swim up. After three hours, the cells were collected with a 10 ml pipette, mixed in one of the plates and checked for agglutination. After 30 minutes of mating, the percentage of QFCs was determined. A minimum of 60% QFCs were required for sufficiently active autolysin. After 45 minutes of mating, the cell suspension was taken off the plates, using a 10 ml pipette, collected in centrifuge tubes and centrifuged at 5000 rpm for 5 minutes in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge. After centrifugation the supernatant was pre-filtered with a 37 mm Glass Fiber Acrodisc Syringe Filter to remove agar and the remaining cells. The liquid was then filter sterilized with a Gelman Sciences VacuCap 0.45 μm filter. The sterile enzyme was stored at –20 °C for up to 6 months.

2.1.2 Insertional Mutagenesis and pSP124S

The sr-u-2-23 *mt*$^-$ strain was used to create fusion defective insertional mutants. This technique creates random insertional mutants (Kindle, 1990). Cells of sr-u-2-23 *mt*$^-$ were grown in 100 ml TAP medium in a 250 ml Erlenmeyer flask for two days under bright light on a New Brunswick gyrotatory shaker. After two days, a small volume of cells (1-10 ml, depending on concentration) was inoculated into 400 ml of TAP medium in a 1000 ml flask. After inoculation, the color of the culture was pale green. Cells were grown two more days at the same conditions, to mid-log phase (1-2 x10^6 cells/ml). Before transformation, the cell concentration was determined by removing 1 ml of liquid from the flask and counting the cells using a hemocytometer. For each transformation 6 x 10^7 log phase cells were used. The proper volume of cells to be used was measured in a sterile graduated cylinder, transferred into sterile 200 ml Nalgene centrifuge bottles and centrifuged at 5000 rpm for 5 minutes using a Sorvall Superspeed RC2-B centrifuge. After centrifugation, the supernatant was discarded, thawed autolysin was added and cells were incubated with the autolysin for 45 minutes at 32 °C in two 50 ml sterile Nalgene Oak Ridge centrifuge tubes. Cells were checked under the microscope to make sure the autolysin degraded the cell walls. When the walls are removed, cells become round and swim more slowly (vegetative cells are more oval then round and they swim faster). After incubation, the cells were centrifuged at 5000 rpm for 5 minutes. The autolysin was discarded and 300 µl of TAP medium was added for each transformation. Cells were transferred to Falcon 5 ml polystyrene round bottom tubes, each containing 0.3 grams of 0.4 mm sterile glass beads (Thomas). The beads had been sterilized in a 220°C

incubator for 2 hours prior to the experiment. 1 μg of BamHI digested pSP124S (Lumbreras et al., 1998; Stevens et al., 1996) was added to the tubes and the suspension was vortexed at top speed for 20 seconds using a Scientific Industries Vortex-Genie K-550-G mixer. After vortexing, the cells were transferred to Nalgene centrifuge tubes containing 20 ml of TAP medium and left under light for 18 hours for recovery and expression of the *ble* gene. The next day, the cells were centrifuged and plated on TAP agar plates containing 15 μg/ml Zeocin™ (Invitrogen) to select transformed colonies. The transformants appear after about 7 to 10 days.

pSP124S has the bacterial *ble* gene which gives resistance to Zeocin™ (Invitrogen). In order to express the exogenous *ble* gene in *Chlamydomonas*, Lumbreras *et. al*. used the endogenous *rbcs2* promoter at the 5' end and also inserted two introns of the *rbcs2* in the coding region of *ble*. This construct was ligated into pBluescriptSK- to create pSP124S.

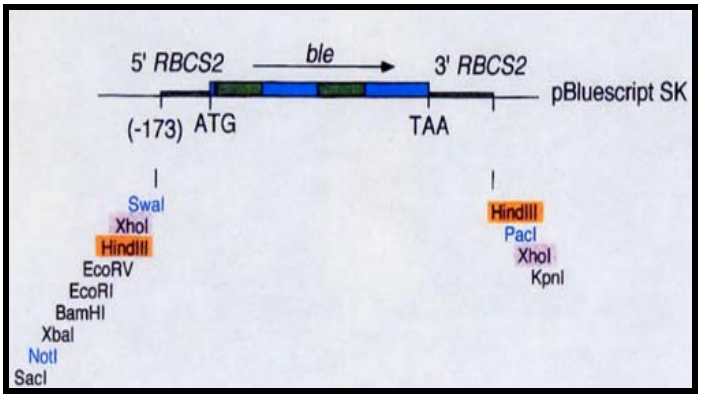


Figure 1. Diagram of pSP124S used in insertional mutagenesis (from Dr. Saul Purton).

2.1.3 Selection of Fusion Defective Mutants by the Streptomycin Selection Method

The streptomycin selection technique was developed by Dr. Forest and Dr. Togasaki (Forest and Togasaki, 1975). We use CC-275, the *sr-u-2-23 mt*$^-$ strain for mutagenesis. This strain has the *sr2* mutation, a uniparentally inherited gene for resistance to 50-500

μg/ml of streptomycin sulfate and high mating efficiency when mated with the opposite mating type. When wild type (mt^+) and *sr2* (mt^-) gametes are mated, all of the zygotes show the mt^+ characteristic, sensitivity to streptomycin, within a short time after gamete fusion (Sager, 1960; Sager and Tsubo, 1961). Segregation of the *sr2* mutation does not follow Mendelian patterns because this gene is located on the chloroplast DNA. This resistance gene can usually only be inherited through mt^+ parental cells. Therefore when we put them in a medium containing streptomycin, after the mating of wild type (mt^+) and *sr-u-2-23* mt^-, all the zygotes and wt m^+ cells will die; only mt^- gametes which did not mate will survive. Thus we can select unmated *sr-u-2-23* mt^- gametes with this procedure.

To select for fusion defective mutants, transformed Zeocin™ -resistant *sr-u-2-23* mt^- cells were grown on 50 μg/ml streptomycin agar plates to maintain their resistance. After 12-15 days of growth (keeping cells on plates for more then 9 days will allow them to form plate gametes if the plates were thin at the time cells were plated), the plates were flooded with SEM-N medium for 2-3 hours to allow the cells to develop flagella and swim up. Cells were taken off the plates and then mated with wt mt^+ gametes for 12-16 hours. After mating, the zygotes form a pellicle on the surface of the medium. The mating mixture was filtered with a sterile 10 μm pore diameter filter to remove these zygote clumps. The filtrate contains the un-mated *sr-u-2-23* mt^- and wt mt^+ cells. Different amounts of the filtrate i.e 10 μl to 100 μl were plated on 200 μg/ml streptomycin plates. In some cases a 1:10 dilution of the filtrate was plated if the mating was not good. If a large zygote pellicle was formed, this meant mating was efficient and dilution was not

necessary. Plates were placed under light for 10 days to allow streptomycin resistant colonies to grow.

2.1.4 Mutant Screening

After the selection, the colonies that grew on streptomycin plates were picked and transferred onto TAP plates for further growth. 16-32 clones were picked from each selection plate. After about 6-7 days on the TAP plates, cells were inoculated into liquid N-free medium for 16 hours and mated with mt^+. The clones that formed pellicles were eliminated. The ones that did not form pellet were retested. Each clone was resuspended in about 200 μl of SEM-N and kept under lights for 16 hours to induce gametogenesis. After gametogenesis, each clone was checked for its motility and agglutination with mt^+. Clone that agglutinate but do not fuse with mt^+ are saved for further study.

2.1.5 Determination of BFC's, Pairs and Groups in the Mutants

Pair and group formation results from mating structure adhesion in mutants that cannot fuse (Forest, 1987). Therefore we can differentiate between adhesion defective and fusion defective mutants by determining if they can form pairs and groups. To do this analysis, the mutants were kept in N-free liquid medium for 16 hours, mated with mt^+ for different durations and fixed with 3 % glutaraldehyde in 10 mM HEPES buffer. The next day, the glutaraldehyde was removed, the cells were washed twice with HEPES buffer and resuspended in HEPES buffer in the original mating volume. Cells were observed under a phase-contrast microscope for BFC, QFC, pair and group formation. When mutants that are capable of mating structure adhesion but not fusion are mated with the

opposite mating type and treated with IKI or glutaraldehyde, the large groups of gametes participating in flagellar agglutination are disrupted. However, the gametes adhering by their mating structures have been shown to form stable pairs and groups that are not disrupted with this treatment (Forest, 1983).

2.2 Genomic DNA Isolation

DNA was isolated with the Qiagen Plant Mini Kit or by phenol-chloroform extraction. In both procedures, cells were grown on solid medium to make sure there was no contamination (in liquid cultures, contamination may not be visible). The Qiagen Kit protocol was followed as described, except cells were not frozen in liquid nitrogen for lysis, because the *Chlamydomonas* cell wall is simpler then higher plants and doesn't require this step. In the phenol-chloroform procedure, several loopfuls of cells were resuspended in 200 μl lysis buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 3% SDS). After resuspending the cells, 4 μl of 100 mg/ml RNase A (Sigma) was added, vortexed briefly, and the cells were incubated in room temperature for 15-20 minutes. After this lysis step, 400 μl TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) was added to increase the volume to make it easier to work in the following extraction steps. 1/10 volume (~60 μl) of 3M sodium acetate (pH 5.2) was also added. Phenol-chloroform extractions were done as follows: 1:1 volume of phenol-chloroform-isoamyl alcohol (Sigma) was added, vortexed 10 seconds, incubated on ice for 5 minutes, centrifuged at 14, 000 rpm for 15 minutes and aqueous layer was taken to a new Eppendorf tube with glass Pasteur pipette. Then the sample was extracted with chloroform (Sigma); the aqueous layer was taken into a new Eppendorf tube again. Chloroform extraction was

done twice to make sure no phenol remained in the sample. To precipitate the DNA, a 1:1 volume of isopropanol was added, the sample was mixed by inverting the tube 5-6 times, incubated on ice for 15 minutes and centrifuged 15 minutes to pellet the DNA. After centrifugation, the supernatant was removed, the pellet was washed with 500 µl 70% ethanol, and centrifuged for10 minutes. Again the supernatant was removed carefully and tubes were laid on the bench to allow the ethanol to evaporate for 5 minutes. After drying the pellet, 50-100 µl of TE buffer or dH_2O was added to resuspend the DNA.

2.3 Southern Blots

pSP124S was used as the template for PCR to generate the *ble* probe. The primers used were named bleforward (5'-atggccaagctgaccagc-3') and blereverse (5'-ggtcgacgtcggttagtc-3'). These primers produce a 538 bp product. A probe for *ori* region of pSP124S was also created with primers ORIAS (5'GGCCAGCAAAAGGCCAGG3') and ORIS (5'GTAGAAAAGATCAAAGGA3'). Radioactive and non-radioactive labeling methods were used in Southern hybridizations. In both methods, running the gel and the blotting steps were the same.

2.3.1 Running the Gel and Blotting

10 µg of genomic DNA isolated from the mutants and the parent strain used for mutagenesis (*sr-u-2-23 mt*$^-$) was digested with KpnI, BamHI or Eco721, ethanol precipitated and dissolved in 30 µl TE. Digested DNA was run on a 1% agarose gel for 4-5 hours by applying 40-50 V. The gel was then subjected to two 15 minutes washes in

alkaline transfer buffer (0.4N NaOH, 1M NaCl) at room temperature by gentle agitation. This step denatures the DNA so it can be fixed to the positively charged membrane in the following blotting step. While denaturing the gel, the membrane was prepared. The positively charged nylon membrane (Roche) was cut in the dimensions of the gel. The membrane was soaked in dH_2O for 5 minutes and then transferred to the alkaline transfer buffer for 5 minutes. The dry blotting method was used for transferring the DNA to the membrane. Assembly of the blot was done as follows: 2 pieces of Whatman blotting paper (Micro Filtration Systems) were placed on a clean baking dish and dampened with alkaline transfer buffer. A corner of the gel was cut (usually the upper left corner) for orientation purpose. The gel was laid upside down onto the blotting papers. This way, the DNA was transferred from the bottom of the gel, which is found to be a more efficient transfer method. A small amount of alkaline transfer buffer was poured onto gel and the membrane was laid on top of the gel (also, the same corner of the membrane was cut before laying it onto the gel). Once the membrane touches the gel it is important that it is not moved, because transfer to the charged membrane starts immediately. A small amount of alkaline transfer buffer was poured onto the membrane and 2 layers of blotting papers were laid on top of the membrane. Finally, at least a few inches of paper towels were placed on top of the blotting papers, and a small weight was placed on top. The DNA was allowed to transfer for 3 hrs. After transfer, the position of the wells was marked with a pencil before the blot was disassembled. The gel was checked to determine if the transfer was complete. The membrane was soaked in 0.4N NaOH for 15 min to fix the DNA. After fixing, the membrane was immersed in 2X SSC for 5-10

minutes to neutralize the membrane. If the hybridization was done in a later date, the membrane was air dried completely and kept in 4°C.

2.3.2 Non-radioactive Method

The DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) was used for the non-radioactive method.

a. Labeling of the Probe

The DNA to be labeled was generated by PCR and gel purified before labeling. 1 μg of purified DNA was added to dH_2O to make 16 μl total volume. The sample was then boiled for 10 minutes and quickly transferred to ice water. DIG-High Prime was mixed thoroughly and 4 μl of it was added to the denatured DNA, mixed and centrifuged briefly. The sample was incubated at 37°C overnight. The next day, the reaction was stopped by heating at 65°C for 10 minutes. It is suggested that 25 ng of labeled probe per ml of hybridization solution should be used for hybridization, but in many cases all of the probe was used.

The labeling efficiency of the probe was checked according to the manual before continuing to the hybridization.

b. Prehybridization

DIG Easy Hyb Granules were dissolved in 64 ml dH_2O to make the hybridization solution. To do this, water was added in two portions, stirred under sterile conditions and kept in a 37°C water bath for 5-10 minutes to dissolve the granules.

Before starting the prehybridizatin, if the membrane was stored, it was soaked in dH_2O before continuing with the prehybridization.

Prehybridization was done in rolling bottles using 10 ml DIG Easy Hyb solution /100 cm^2 membrane at 37-42°C in a hybridization oven for 30 minutes.

c. Hybridizaton

During the prehybridization, the hybridization solution was prepared as follows: The DIG labeled DNA probe was denatured by boiling for 5 minutes in a boiling water bath. After boiling, the probe was transferred quickly to ice water to prevent renaturation. DIG Easy Hyb solution was also heated to 68°C during the prehybridization step. 25 ng of probe/ml DIG Easy Hyb solution was added to the heated DIG Easy Hyb solution, mixed gently to prevent foaming (in many cases 1 μg labeled probe in 10 ml hybridization solution was used to get signal).

After the prehybridization step, the prehybridization solution was taken off and the probe-Easy Hyb solution was added to the bottle. Hybridization was done at 68°C over night in a hybridization oven.

The next day, the hybridization solution was poured into a sterile Falcon tube and kept in -20°C. (The hybridization solution can be used 3-4 times.)

d. Post Hybridization Washes:

The membrane was washed with an ample amount (~250 ml) of 2X SSC, 0.1% SDS twice for 15 minutes at R/T by gentle agitation on a shaker. It was then washed with 0.5X SSC, 0.1% SDS twice for 15 minutes at 68°C in a hybridization oven.

e. Immunological Detection:

The membrane was rinsed with ~ 25 ml of washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5 and autoclaved or filter sterilized; 0.3% v/v Tween 20 was added after the buffer was made) for 1-5 minutes at R/T. (All the washes below were done at R/T by

gentle agitation). After this brief washing, the membrane was incubated in 100 ml 1X blocking solution for 30 minutes. 1X blocking solution was made by 1:10 dilution of 10X solution which is supplied in the kit with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; adjust pH with solid NaOH and filter sterilize). Then the membrane was incubated with 20 ml of antibody solution (1:10 000 dilution of Anti-Digoxigenin-AP with blocking solution) for 30 minutes. After incubation with antibody, the membrane was washed twice with 100 ml washing buffer for 15 minutes. The membrane was equilibrated in 20 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5) for 2-5 minutes.

For exposure, the membrane was laid DNA facing up over a glass plate, 1 ml CSPD ready to use was spread evenly over the membrane and immediately covered with saran wrap or acetate film to prevent drying of the membrane. The membrane was incubated for 10 minutes at 37°C and then kept in R/T for 20 minutes. The results were recorded with the FluorChem^{TM} 8900 Imager (Alpha Innotech). If the membrane was exposed to X-Ray film, after incubation with CSPD, the X-Ray film was laid over the membrane in a dark room and kept in a development folder. The membrane was exposed to film for 15-60 minutes and the X-Ray film was then developed in a dark room.

2.3.3 Radioactive Method

The procedure was taken from Dr. Benjamin Ortiz's lab.

a. Radioactive Labeling of the Probe

The DNA to be labeled was generated by PCR and gel purified before labeling, as in the non-radioactive labeling method. Around 100 ng purified DNA was added to dH_2O in 21

µl total volume. The probe was denatured at 95°C for 5 minutes and transferred to ice water. RadPrime DNALabeling System (Invitrogen) was used for labeling. Quickly, 3 µl of dNTP mix and 20 µl of 2.5X buffer were added at the bench. After this, 1 µl of Klenow and 5 µl of α-^{32}P dCTP were added behind a safe shield. The contents were mixed and centrifuged briefly. The sample tube was then placed into a radioactive shielding box and incubated at 37°C for 45 minutes.

After the labeling was finished, the probe was cleaned using a Micro Bio-Spin Chromatography Column (Biorad). To do this, the column was inverted several times to resuspend the gel inside of it, and the column tip was broken and placed in a collection tube. Then the top cap was removed to allow the excess buffer to flow out. The drained buffer was discarded and the column was put back in the collection tube and centrifuged at 1000 g for 2 minutes to remove the remaining buffer. The column was transferred to a clean microcentrifuge tube; the sample was loaded onto the column, and centrifuged at 1000 g for 4 minutes. The eluted sample was now in SSC or TE buffer depending on the choice of column.

The efficiency of labeling was determined with a Scintillation Counter (Technical Association Surface Monitor Model TBM-15). Briefly, 2 µl of probe were spotted onto a Whatman paper in a glass tube. Counting was done in this glass tube. Usually 600, 000 counts per minute (cpm) were measured. The probe was denatured right before starting the hybridization step by heating at 95°C for 5 minutes.

b. Prehybridization

5-10 ml room temperature Quick Hyb solution (Startagene) and herring sperm DNA (Invitrogen) were used for prehybridization. Quick Hyb solution was taken from 4°C and

brought to R/T before adding. 100 µl of herring sperm DNA (10 mg/ml stock concentration) was denatured in a few ml of Quick Hyb solution at 95°C for 5 minutes and added to hybridization bottle with the Quick Hyb solution. The membrane was incubated in rolling bottle at 68°C for 60 minutes.

c. Hybridization

0.5-1 ml Quick Hyb solution was taken from the bottle and added to the denatured probe. All of the probe mix was then added back to hybridization bottle and hybridization was allowed to proceed at 68°C for 2 hours.

d. Post Hybridization Washes

After 2 hours, the hybridization solution was discarded in the radioactive waste container. The membrane was rinsed twice with 2X SSC, 0.1% SDS, and then washed once with 2X SSC, 0.1% SDS at R/T for 15 minutes. It was then washed again with 2X SSC, 0.1% SDS at 65°C for 15 minutes. After each wash, the membrane and wash solution were checked to determine if any radioactivity was remaining (Technical Association Surface Monitor, Model TBM-15).

e. Radioactive Detection

The membrane was exposed to X-Ray film for 2 hours or exposed to a phosphorimager.

2.4 Sequencing the Flanking Genomic Region of the Insertion

2.4.1 TAIL-PCR and RESDA-PCR

TAIL-PCR (Thermal Asymmetric Interlaced PCR) (Dent et al., 2005) and RESDA-PCR (Restriction Enzyme Site-Directed Amplification PCR) (Gonzalez-Ballester et al., 2005) were used to sequence the flanking genomic region of the insertions in the mutants.

Primers designed for the insert (gene specific primers) and degenerate primers that will bind to the genomic DNA were used to amplify the flanking regions of the insertions. Schematic representation of TAIL-PCR is shown in Figure 2.

0.5-1 μg of undigested genomic DNA was used as the template for the primary PCR reaction. The product of the primary PCR is diluted 1:10 to 1:25, depending on the amount of the product, and used as template for the secondary PCR reaction along with nested gene specific primer and degenerate primer for TAIL-PCR; in RESDA-PCR, primer Q0 (same sequence of 5' end of degenerate primers used in the primary reaction) was used in the secondary and tertiary PCR. After secondary PCR, tertiary and sometimes more nested PCRs were done until the product became specific enough (no smear, clear band) so it could be gel purified and sequenced. In some cases, if there was not a single band produced after tertiary PCR, a band that is thought to be more specific among others was cut off the gel, purified and the sample was used as the template for the following nested PCRs.

The degenerate primers used in TAIL-PCR are completely degenerate, whereas in RESDA-PCR the primers are partially degenerate. RESDA-PCR primers have restriction site sequences and a limited number of degenerate sequences at the 3' end to allow the primer to bind at various restriction sites in the genome. This 3' region is connected to the 5' end, which has a fixed sequence, by several inosine bases which also give more degenerate characteristic to the primer. The 5' fixed sequence was used in the following nested PCR after doing the first round of PCR to get a specific product. The list of primers is shown in Table 1.

Table 1. List of Primers used in TAIL-PCR and RESDA-PCR

Primer Name	***Sequence***
Degenerate Primers	
RD 227	5'-NTCGWGWTSCNAGC-3'
Eco721	5'-CCAGTGAGCAGAGTGACGIIIIINNS*CACGTG*[S~Q]-3'
Q0*	5'-CCAGTGAGCAGAGTGACG-3'
Gene Specific Primers	
Ble1	5'-TGTTGTCCGGCACCACCTGGTC-3'
Ble2	5'-CTGATGAACAGGGTCACGTC-3'
BleC	5'-AGATGTTGAGTGACTTCTCTT-3'
RD223	5'-TTGGCTGCGCTCCTTCTGGCATTTAAATC-3'
RD224	5'-GCATTTAAATCTCGAGGTCGAC-3'
RD225	5'-GATAAGCTTGATATCGAATTCC-3'

*Q0 is not degenerate, but it is used in pair with gene specific primers

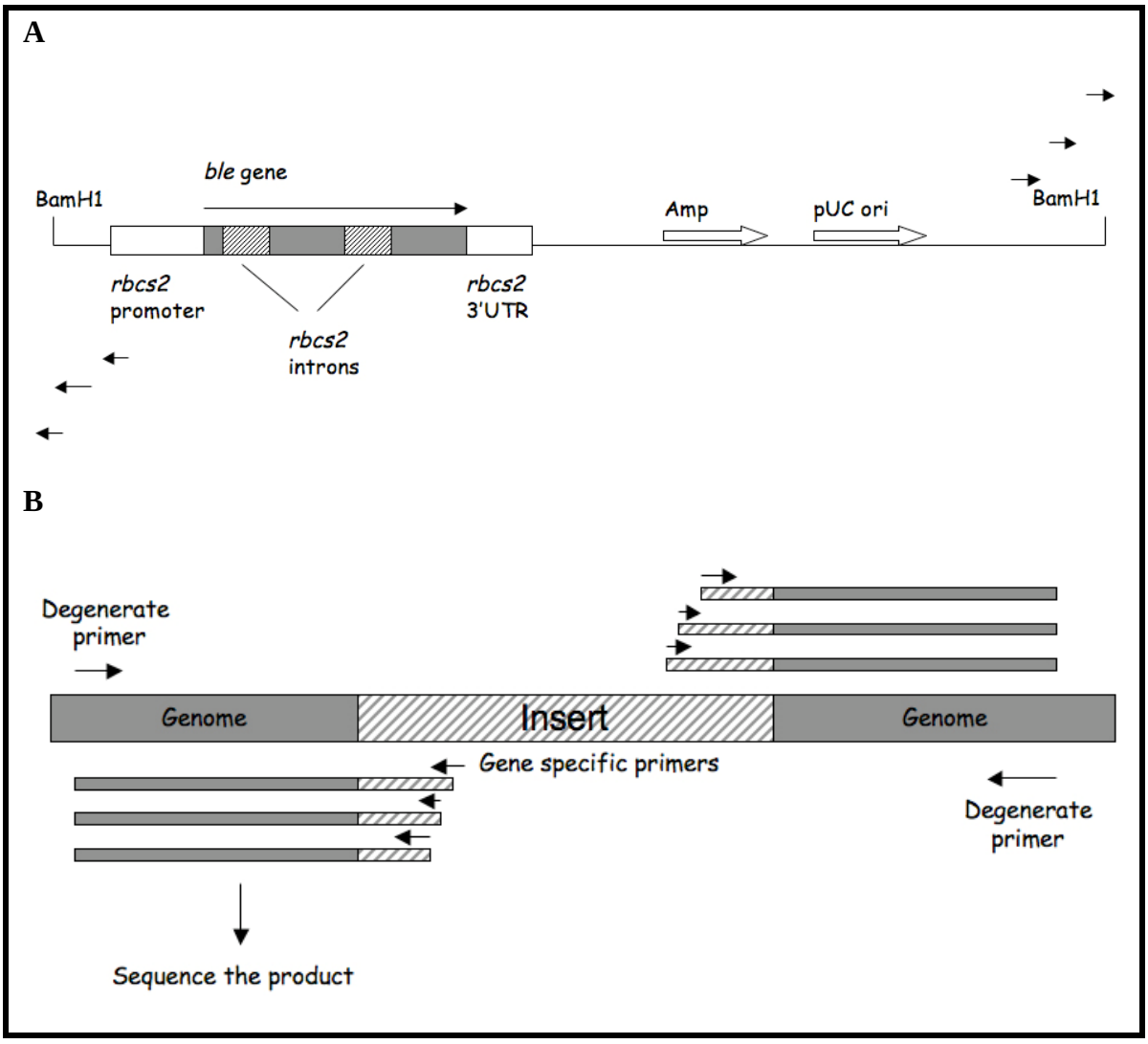


Figure 2. Schematic Explanation of TAIL-PCR and RESDA-PCR. A. Diagram of BamHI digested pSP124S. Possible insertion into the genome and position of gene specific primers at the 5' and 3' end of plasmid. Primers that bind to second exon of *ble* were also used but not shown here. **B.** Mechanism of TAIL-PCR and RESDA-PCR. Gene specific and degenerate primers produce more specific product after each nested PCR shown by smaller product being produced after each reaction.

2.4.1.1 PCR Thermal Cycling Conditions

Thermal cycling conditions were adapted from Dent et al., 2005, with minor changes. The amplification duration was increased to 3 min and the annealing temperature was changed according to the T_m of the gene specific primer used in any reaction.

PCR Thermal Cycling Conditions:

Reaction	Step	Thermal Settings	No. of Cycles
Primary	1	95°C, 5 min	1
	2	94°C, 1 min; 62°C, 1 min; 72°C, 3 min	5
	3	94 °C, 1 min; 25°C, 3 min ramping to 72°C over 3 min; 72°C, 3 min	1
	4	94 °C, 30 s; 68°C, 1 min; 72°C 3 min; 94 °C, 30 s; 68°C, 1 min; 72°C 3 min; 94°C, 30 s; 44°C, 1 min; 72°C 3 min	15
	5	72°C, 5 min	1
Secondary	1	94 °C, 30 s; 64°C, 1 min; 72°C 3 min; 94 °C, 30 s; 64°C, 1 min; 72°C 3 min; 94 °C, 30 s; 55°C, 1 min; 72°C 3 min;	12
	2	72°C 5 min	1
Tertiary	1	94 °C, 30 s; 55°C, 1 min; 72°C 3 min;	30
	2	72°C 5 min	1

2.4.1.2 Assembly of PCR Reactions

Epicentre Biotechnologies' FailSafe PCR PreMix and the FailSafe Enzyme Mix were found to be the best reagents. The FailSafe Selection Kit was first tested and Mix E was found to be the best buffer for *C. reinhardtii* genomic DNA. After determining this, Mix E along with the FailSafe Enzyme Mix was used in all PCR reactions.

Different primers concentrations were tested and 5 pmol gene specific primer and 50-60 pmol degenerate primer concentrations were found to be the best, as suggested (Dent et al., 2005; Gonzalez-Ballester et al., 2005).

Assembly of a Primary PCR for 1 Reaction Tube in 20 µl Total Volume:

10 µl Mix E

5 µM Gene Specific Primer

50 µM Degenerate Primer

0.2 µl Enzyme Mix (1.25 units)

1 µg Genomic DNA Template

X µl dH_2O

Assembly of Secondary PCR Reaction in 20 µl Total Volume:

10 µl Mix E

5 µM Nested Gene Specific Primer

50 µM Degenerate Primer or Q0 primer for RESDA

0.2 µl Enzyme Mix (1.25 units)

2 µl of diluted Primary PCR Product

X µl dH_2O

Assembly of Tertiary PCR Reaction in 20 µl Total Volume:

10 µl Mix E

5 µM Nested Gene Specific Primer

50 µM Degenerate Primer or Q0 primer for RESDA

0.2 µl Enzyme Mix (1.25 units)

2 µl of diluted Secondary PCR Product

X µl dH_2O

2.4.1.3 Genome Walking from the Insertions

Primers BleC (5'-AGATGTTGAGTGACTTCTCTT-3') and RD223 (5'-TTGGCTGCGCTCCTTCTGGCATTTAAATC-3') which bind to pSP124S and primers NSG3R2 (5'-CCGTGCTTACTGACTGCAAC-3') and NSG3R4 (5'-tttgccatatggttgctctg-3') which bind to scaffold-44 were used for genome walking at the clone-5 insertion site. Primers J1-F (5'-CAGTGCTGACCCCCACTATT-3') and RD225 (5'-GATAAGCTTGATATCGAATTCC-3') were used for walking into the genome from the J1 insertion site.

2.4.1.4 Determining if there is a Possible Deletion in the Insertion Region of J1

Primers J1-F (5'- cagtgctgacccccactatt-3') and J1-R (5'- cttcacgtcaactccccatt-3') were used to amplify genomic DNA upstream of the insertion point. Primers j1-f2 (5'-CGTTGAACATAGCGACCTCA-3') and j1-r2 (5'- CCTGCATCGCATACACTGTC-3') were used to amplify the downstream region of the insertion. Primers j1-f3 (5'-CATCACGACCCTCATGTTTG-3') and j1-r3 (5'- GGCAGGACAGATAGGAGCAG-3') were used to amplify a region of downstream gene model C_37000110.

2.4.2 Additional Methods Used to Identify the Insertion Site

2.4.2.1 Plasmid Rescue

We digested the genomic DNA isolated from the mutants and *sr*-, and recircularized it with DNA ligase (New England Biolabs). 500 ng of digested DNA was used for re-circularization. (In the circularized pieces we should have vector sequence and some genomic DNA, which will help us identify the gene) (Tam and Lefebvre, 1995). This ligated DNA was then used to transform *E.coli* which was selected on ampicillin plates.

The plasmid was then isolated with the Qiagen plasmid mini kit and sent for sequencing.

2.4.2.2 Ligation Mediated Suppression (LMS) PCR

LMS-PCR was done to sequence the flanking genomic region of the insertions (Strauss et al., 2001). Briefly, genomic DNA was isolated and 3-5 μg was digested with Eco721, which does not cut in the pSP124S. The digest was purified with phenol-chloroform and dissolve in 20 μl of dH_2O.

Next, adapters were ligated to digested DNA as follows. The upper adapter (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3') and lower adapter (5'- ACCTGCCC{AMC7~Q} -3') stock solutions were 100 μM. Prior to use, equal volumes of adapters were mixed, boiled for 5 minutes and cooled to room temperature. 5 μM adapters were ligated to 10 μl of digested DNA using 10 units of T4 DNA ligase (Bioline), overnight at 16°C.

The ligation was diluted 1:5 and 1 μl was used for primary PCR. For primary PCR primers, AP1 (5'-CTAATACGACTCACTATAGGGC-3') which binds to upper adapter sequence and sense primer 4ble (5'-ggtcgacgtcggttagtc-3') were used. The primary PCR product was diluted 1:100 and 1 μl was used for nested PCR (secondary PCR). For nested PCR, primers AP2 (5'-AATAGGGCTCGAGCGGC-3') and ble-specific primer2 (5'-atggccaagctgaccagc-3') were used.

2.5 Complementation of the Mutants and the BACs

The *Chlamydomonas* BACs used for complementation were obtained from the Clemson University Genomics Institute. BAC 33A3 contains the 3 gene models adjacent to the insertion region in mutant clone-5 at scaffold-44. These gene models are C_440054, C_2470009 (annotated only in version 2 of the *Chlamydomonas* genome), and e_gwH.44.48.1 (protein ID 106481). BAC 37P6 contains the gene models at the insertion site of mutants J1 at scaffold-37. These gene models are C_370109 and C_370024.

Three approaches were taken for these complementation experiments: (1) transforming cells with the unmodified BACs, (2) co-transformation of the BACs with pHyg3 and (3) modification of the BACs by inserting a selectable marker for direct selection of transformants after BAC transformation.

2.5.1 Transformation with Unmodified BACs

J1 was transformed with circular unmodified BACs (5-10 μg) and transformants were plated on TAP plates.

2.5.2 Co-transformation

5-10 μg of circular BAC and 1-3 μg of pHyg3 (Berthold et al., 2002), circular or linearized with EcoRV was co-transformed into the mutants by the glass bead method. Transformants were selected on 10 μg Hygromycin (Genlantis, CA) TAP plates.

2.5.3 Transformation with Modified BACs and Generation of Recombinant BACs

The Quick and Easy BAC Modification Kit (Gene Bridges, Heidelberg, Germany) was used to insert selectable markers into the BACs.

BAC modification was done as follows:

2.5.3.1 Generation of the Insert

The plasmid Hyg3 contains the *aphVII* cassette for hygromycin B resistance (Berthold et al., 2002). The Hyg3 cassette was cut out of from the original vector that was used (pUCBM20) by digesting pHyg3 with EcoRV and KpnI. The cassette was gel purified and ligated into pBluescript SK-. pSP124S described above, was also digested with EcoRV and KpnI to produce a pBluescript that had the same restriction ends as the Hyg3 cassette. This digestion released the *ble* cassette from pSP124S. Digestion was run on the gel and pBluescript was cut out of the gel and purified. This purified vector was ligated to the Hyg3 cassette using T4 DNA ligase supplied with the pGEMT Easy Vector System (Promega). The vector to insert (Hyg3 cassette) ratio was 3:1 in ligation reaction. JM109 competent cells (Promega) were transformed with 2 µl of the ligation, and transformants were selected on 100 µg/ml ampicillin containing LB plates. The plasmid was isolated with Qiagen Mini Kit and digested with NotI (which cuts once) to determine its size and to make sure ligation happened correctly. This new plasmid was named pHyg3MA to differentiate it from the original pHyg3. To make sure the Hyg3 cassette was still functioning, pHyg3MA was used to transform *sr-u-2-23 mt-* cells and many resistant colonies were formed. pHyg3MA was transformed in both circular and linear form (by digesting with XbaI). The linear plasmid had a higher efficiency then the

circular plasmid.

Inserting only the *Hygromycin* cassette into the BACs wouldn't allow us to select modified BACs in *E. coli*, because the cassette has a *Chlamydomonas* promoter which will be expressed only in *Chlamydomonas.* In order to select for the modified BACs, we needed a dual expression cassette (a cassette that can be expressed both in *Chlamydomonas* and *E. coli*), but there weren't any in existence. To produce such a cassette, we ligated a prokaryotic expression cassette next to the *Hygromycin* cassette in pHyg3MA. The Tn5-neomycin cassette, which was included in the BAC modification kit, was used for this purpose. This cassette was amplified by PCR using Epicentre reagents and the purified product was ligated into a pGEMT Easy vector (Promega). 2 μl of the ligation product was used to transform *E.coli* as described in the kit manual and transformed colonies were selected on LB plates containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The plasmid was isolated using Qiagen Plasmid Mini Kit and the Tn5-neomycin cassette was excised with NotI. The cassette was gel purified and ligated into NotI digested, gel purified pHyg3MA. This ligation was again used to transform *E. coli* and transformed *E. coli* was again selected on LB plates containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. This plasmid was named pHyg3MA-Tn5neo. pHyg3MA-Tn5neo was used as a template for PCR for creating the selectable marker insert (*Hygromycin* and Tn5-neomycin fusion cassettes) to be used for modification of the BACs. To create the insert, 50 bp homology arms were added to the 5'end of the primers. One of the primers was T7 (5'-GTAATACGACTCACTATAGGGC-3') primer. The other primer binds at the opposite site of MCS of pBluescript SK- and was named B-Gal-f (5'-ATGACCATGATTACGCCAAGC-3'). The sequences of the oligos with the

homology arms are:

NARHA-T7:

5’GAATTCCCCGGATCATGCCGCCAGTAGCACGAGTGAAGAGGTTCATGAGG-GTAATACGACTCACTATAGGGC-3’

NARHA-BGal:

5’-CGCAGCGATTTACCCGTGTTGCGGAAATAGAGCGTGTCGGCAGTGGCGTA-ATGACCATGATTACGCCAAGC-3’.

The underlined regions are the homologues regions to the *nrt2* gene that were inserted into the BACs when they were created at the LeFebvre Lab at the University of Minnesota. The purpose of the insertion of the *nrt2* gene was to use it as a selectable marker for their experiments; because many *Chlamydomonas* researchers use CC124 or CC125 wild type strains to create mutants. These two strains cannot transport nitrate and therefore cannot grow on nitrate as a sole nitrogen source. The *nrt2* gene encodes a subunit of nitrate transporter. When the BACs are put into the cells they become competent and can grow with nitrate.

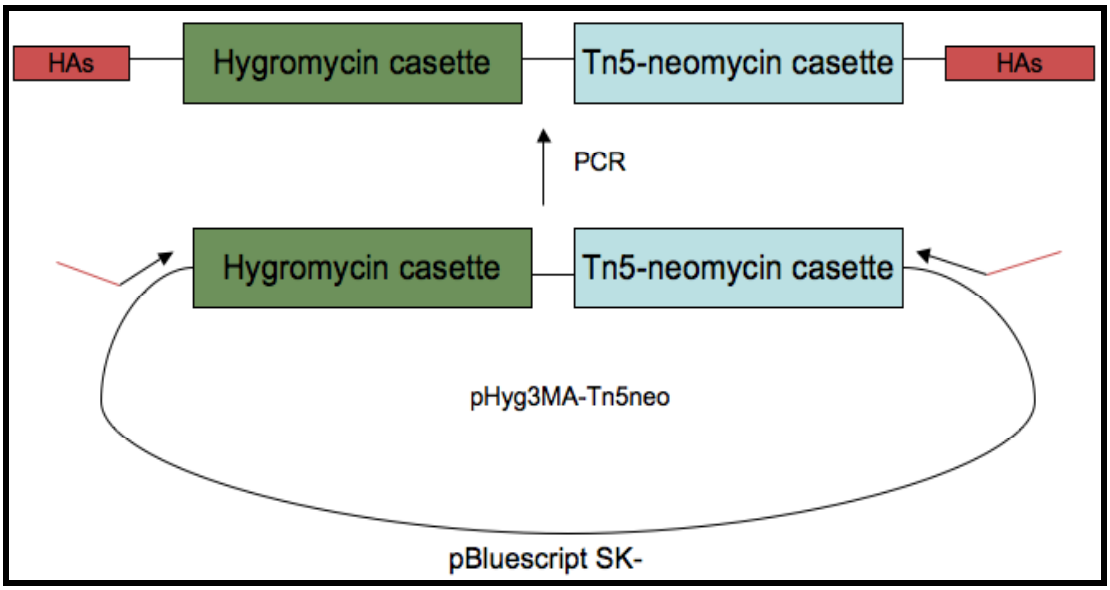


Figure 3. Diagram showing the creation of the *Hygromycin*-Tn5neomycin fusion cassette with homology arms.

2.5.3.2 PCR Conditions

Phusion Hot Start DNA Polymerase (New England Biolabs), which has very high accuracy, was used to create the insert with homology arms. Epicentre FailSafe Enzyme Mix was more efficient then the Phusion Polymerase, but its fidelity was lower (because the Epicentre enzyme mix contains non-proof reading enzyme also). Both enzymes also produced very small amount of product with oligos which had homology arms. To make the insert with homology arms, first T7 primer and a primer called BGal-f that binds to the opposite site of the MCS of pBluescript were used to amplify the fused *Hygromycin* and Tn5-neomycin cassettes. Cycling conditions for this reaction was 98°C 2 minutes (hot start) then 30 cycles of 98°C for 10 seconds, 58°C for 30 seconds and 72°C for 1 minute followed by a 5 minutes extension at 72°C. This 3 kb product was then diluted 1:10 and used as a template with the homology arm primers (NARHA-T7 and NARHA-

BGal) to make the insert. Cycling conditions were 98°C for 2 minutes (hot start) then 30 cycles of 98°C for 10 seconds, 58°C for 30 seconds and 72°C for 1½ minutes followed by 5 a minute extension at 72°C. In both reactions HF buffer was used with Phusion Hot Start DNA Polymerase (New England Biolabs), because HF buffer has higher fidelity comparing to GC buffer. DMSO was added to the second PCR with homology arm primers to get more product. Without DMSO the amount of product was very small. The PCR product was digested with DpnI, which will destroy the circular plasmid template, but not the PCR product. After digestion, the DpnI was heat inactivated and the PCR product was gel purified. This gel purified sample was used for recombination.

2.5.3.3 Quick and Easy BAC Modification Protocol

Before starting the experiment, bacteria that contain the BACs to be modified were streaked on LB plates containing 15 μg/ml chloramphenicol. The control BAC that was included in the kit was also freshly streaked.

On day 1 of the experiment, 2 colonies (only one will be used for each BAC recombination, the other started as back-up) were picked from each of the experimental BACs (BAC 33A3 and BAC 37P6) and from the control BAC and were grown overnight in 1 ml LB medium containing 15 μg/ml chloramphenicol in a lid punctured microcentrifuge tube with shaking.

On day 2 of the experiment, the pRED/ET was electroporated into the bacteria grown overnight. Before starting, ddH_2O and electroporation cuvettes were chilled at least two hours on ice. A benchtop centrifuge was kept in 4°C for the duration of the experiments. To start the experiment, 30 μl of overnight grown bacteria were inoculated into 1.4 ml

LB containing 15 μg/ml chloramphenicol in lid punctured microcentrifuge tubes. Cultures were grown for 2-3 hours with shaking at 200 rpm (the kit suggested 1000 rpm but 200 rpm also worked). After growing the bacteria, cells were centrifuged at 11 K rpm in the chilled centrifuge for 30 seconds, the supernatant was tipped off quickly and the cells were resuspended in 1 ml chilled dH_2O. The cells were centrifuged, the supernatant taken off and the cells were resuspended in 1 ml chilled dH_2O again. The cells were centrifuged again and supernatant was removed. After this last wash, 20-30 μl dH_2O remain in the tubes. The cells were resuspended in this remaining dH_2O and kept on ice. 1 μl of pRED/ET was added to each tube, mixed gently by flicking and the suspension was transferred to chilled 0.1 cm electroporation tubes (Invitrogen). Electroporation was done using the BioRad Gene Pulser electroporator. Settings were 1.8 kV, 200 Ohms and 25 μF. After electroporation, the cells were resuspended into 1 ml LB without any antibiotics and grown at **30°C** with shaking for 70 minutes. 100 μl of the cells were plated on LB plates containing 15 μg/ml chloramphenicol, 3 μg/ml tetracycline and were grown overnight at **30°C**. It is important to wrap the plates with foil and keep the plates in the dark because tetracylin is light sensitive.

On day 3 of the experiment, 2 colonies (one as back-up) were picked from each plate and inoculated into 1 ml LB, 15 μg/ml chloramphenicol plus 3 μg/ml tetracycline in lid punctures microcentrifuge tubes and grown overnight at **30°C** in the dark.

On day 4, the insert (*Hygromycin* Tn5-neomycin fusion cassette) was electroporated into pRED/ET transformed bacteria. Again material was chilled as described above before starting. Two cultures (one for induced for the uninduced experiment) of each BAC strain were inoculated with 30 μl of overnight cultures in 1.4 ml LB, 15 μg/ml

chloramphenicol plus 3 μg/ml tetracycline and grown in dark with shaking for 2 hours at **30°C**. For the induced experiments, 50 μl of 10% arabinose was added to the tubes and all the cultures (induced or uninduced) were grown 1 hour at **37°C**. This step will induce the expression of the recombination proteins. After inducing, the cells were electroporated, this time with selectable marker cassettes using same electroporation conditions. For electroporation of the *Hygromycin* Tn5-neomycin fusion cassette ~500 ng DNA was used. For the control insert 1 μl was used as suggested. After electroporation, cells were grown in 1 ml LB without any antibiotics for 2 hours at 37°C. This is the step where recombination occurs. After this, 100 μl of each culture was plated on LB, 15 μg/ml chloramphenicol plus 15 μg/ml kanamycin plates and grown overnight at 37°C. The colonies growing on kanamycin should have recombined correctly, but this needs to be verified.

2.5.3.4 Verification of Recombination

Restriction analysis was done with KpnI and BglII. The BACs were also analyzed by PCR to verify the recombination.

The PCR for the kanamycin cassette was performed with primers:

Tn5-neo-f (5'- TGGACAGCAAGCGAACCGGAATTGC-3') and

Tn5-neo-r (5'- TCAGAAGAACTCGTCAAGAAGGCG-3').

The primers for amplification of the Hygromycin cassette were:

Hyg3-f2 (5'- AGAGCACCAACCCCGTACT-3') and

Hyg3-r2 (5'- GAAGGCGTTGAGATGCAGTT-3').

PCR to show correct replacement was done with primers:

f-NarReplace (5'-GTCCTTGATGCCCTGGTAAA-3')

r-NarReplace (5'-TTTGCACTCATCGAAAGCAC-3')

NarCh-f-2091 (5'-gaacgaaacagtcgggaaaa-3')

NarCh-r-3552 (5'-TGATGACCTGTTCCTCACCA-3')

pBACmn6020 (5'-GGCCCAAGTATTCGCTATCA-3')

2.5.3.5 Transformation of Mutants with Modified BACs

5 μg of circular modified and unmodified BACs were used to transform the mutants. Unmodified BAC 33A3 (as the negative control) and the modified version of it, BAC 33A3 clone 5-4, were used to transform mutant cl-5. Mutant J1 was transformed with unmodified BAC 37P6, and modified clones 5-9 and 5-4. Transformants were selected on TAP hygromycin 10 μg/ml plates. Cl-5 was also transformed as a positive control with circular pHyg3 to determine the transformation efficiency with the circular plasmid.

2.5.3.6 Presence of BACs in Transformed Mutants

We do not have evidence if BACs are completely inserted into the *Chlamydomonas* genome (Dr. Pete Lefebvre, personal communication). We did PCRs for different regions of the BACs in the BAC transformed mutants. After BAC transformation, colonies growing on hygromycin plates were picked and transferred to new plates for further growth. DNA was isolated from the transformants and from the untransformed mutants and PCR was performed with primers that bind to different regions of the BAC.

2.6 Determination of Rescue of the Fusion Defective Phenotype in J1

The mutant J1 was transformed with BACs to rescue the fusion defective phenotype. As described above, transformations were performed with either unmodified (UM) BAC 37P6, unmodified BAC 37P6 plus pHyg3 or the modified BAC which was named BAC 37P6 5-9. For transformations using unmodified BACs, the cells were plated on TAP plates and allowed to grow for about one week. After one week, the plates were flooded with nitrogen free medium and the cells were allowed to grow flagella for 2-3 hours. The cells were collected from the plates and transferred to tubes and kept in nitrogen free medium for 16 hours to induce gametogenesis. Following this induction, 3 ml of the cells were mated with 3 ml of mt^+ cells for at least 5 hours. After mating, the cells were plated onto 4% TAP plates. The zygote plates were kept in the dark for 6 days to allow them to mature. After maturation, vegetative cells were gently scraped off of the surface and cells remaining on the plates were chloroformed (subjected to chloroform vapor) for 45 seconds. The plates were then left in the light and checked for zygote germination. If the transformants were selected, either by co-transformation or transformation with modified BACs, the transformants were screened for mating ability.

2.7 Genetic Mapping with Temperature Sensitive Mutants

The previously isolated temperature sensitive mutant, *gam-10* (Forest, 1983) was crossed to mapping strains (strains with different genetic markers). These strains were obtained from the *Chlamydomonas* Culture Collection. The mapping strains and the genetic markers they carry are shown in Table 2. General mapping techniques were followed as shown in the *Chlamydomonas* Sourcebook (Harris, 1988). To do the mapping, *gam-10*

mutant was mated with each of these strains at room temperature for 1 hour and the mating mixture were spread onto 4% TAP plates. These zygote plates were kept under light for 1 day and then kept in the dark for 6 days. After this maturation step, zygotes were aligned onto non-selective medium, chloroformed for 45 seconds, and kept under light for 1 day. Next day, germinated zygotes were dissected under the dissection microscope. Progeny were allowed to grow for about 1 week. When the colonies were grown, they were picked and transferred to non-selective medium. Then each zygospore colony progeny was transferred to scoring (selection) plates. To do this, cells from each colony was suspended in liquid TAP or N-free medium and 5 µl was spotted onto indexed selection plates. The second step in genetic mapping is determination of the fusion defective phenotype in the progeny. To do this, cells from each of the progeny were mated with wild type mt^+ and mt^- at room temperature to determine its mating type. After mating type was determined, mating type minus progeny were mated at the restrictive temperature (32°C). To determine the linkage of the genetic markers and fusion defective phenotype (*gam-10*), each marker and the fusion defective phenotype were scored.

Table 2. Mapping strains and media used for genetic mapping

Strain	**Media**
CC 27	TAP + 4 µg/ml Nic: Scoring of Thi requirers. Minimal + 5 µg/ml Thi + 4 µg/ml Nic: Scoring of ac31 TAP + 5 µg/ml Thi: Scoring of Nic requirers YTAP + 5 µg/ml Thi : Non-selective medium for all
CC 641	TAP + 5-20 µg/ml Cyc + 4 µg/ml Nic: Scoring of Cycloheximide^R TAP: Scoring of Nic requirers. TAP + 4 µg/ml Nic: Non-selective medium for all
CC 1768	Minimal + add 0.292 g glutamine in 5 ml dH_2O (filter sterilized) after autoclaving media

2.8 Scanning Electron Microscopy

2.8.1 Dehydration and Critical Point Drying

Cells were mated for 15-20 minutes and fixed with 1:1 volume of 3% glutaraldehyde in 10 mM HEPES, pH 7, overnight at 4°C. The glutaraldehyde was prepared a day before the fixation. The glutaraldehyde was gently dropped down the side of the tubes to prevent disruption of the adhering mating pairs. The next day, the glutaraldehyde was removed, and the cells were washed twice with HEPES buffer and finally resuspended in the original volume of HEPES. During the washes, the cells were centrifuged at a low speed to prevent breaking of the pairs.

Dehydration

Cells were dehydrated on Nuclepore filters (13 mm diameter, 0.8 μm pore size) in Millipore filter holders. 2-3 drops of fixed cells were used for each sample drying; (too many cells can block the filter and visualization of cells packed too close together is also be difficult. After adding the cells to the filter, the cells were gently pushed down with a syringe and immediately dehydration was started by adding several drops of 30% ethanol into the filter holder. It is important that the cells are never allowed to get dry during the process. The 30% ethanol was allowed to drip for 5 minutes, while continuously adding more. After 5 minutes in 30% ethanol, the liquid was gently pushed down again with a syringe and 60% ethanol was added. Cells were kept in 60% ethanol for 10 minutes. After 10 minutes, the liquid was pushed down (if necessary) and 90% ethanol was added for 10 minutes. After this, 100% old ethanol was used for 10 minutes. As the alcohol concentration increases, the flow rate of the alcohol increases and it becomes important to check the samples carefully to make sure they don't get dry. After these washes, the

filters were removed from the filter holders in a Petri dish of 100% ethanol and wrapped in labeled aluminum foil packets with holes to allow exchange in the critical point dryer. These packets have been labeled. The filters are put into the packets with the cells facing down; so the cells are facing the labeled face of the foil. The foil was folded over the cells. After folding, the foil packets were transferred to a new Petri dish filled with 100% ethanol and kept there for 10 minutes (this can be done for 5 minutes if necessary). Then the packets were transferred twice into fresh (newly opened) 100% ethanol for 10 minutes each. Again, the packets must be transferred quickly to make sure samples don't get dry. If necessary, the last step can be extended to 30 minutes before continuing to critical point drying.

Critical Point Drying

Critical point drying was done in a Tousimis 790 critical point dryer.

Loading

After opening the chamber, the power button is pressed. First the chamber was filled half way with 100% ethanol (taken from the Petri dish with the foil packets). Then the packets were added quickly. The chamber lid was replaced by hand-tightening the nuts.

Cooling

After placement of the samples, the CO_2 tank valve was opened very slightly.

The cooling button is pressed; when the temperature reached 0°C, cool was swithed off.

Chamber Filling

Pressing the fill button causes the pressure to rise to equal the pressure in the CO_2 tank and fills the chamber with CO_2. The cool button is used to maintain the chamber at a temperature between 0°C and 2°C.

Purge (Exchange of Ethanol with Liquid CO_2)

When the chamber is full, the ethanol is replaced by the CO_2 by purging (for about 8 minutes). During this time CO_2 is allowed to enter the chamber and the ethanol and CO_2 exit the chamber; the pressure remains at 750-850 psi. Before ending the purge, we make sure no more ethanol is remaining the chamber by smelling the exhaust liquid. To end purge, we first stop cool, fill and purge. We then press fill for 15 seconds to make sure chamber contains the maximum amount of liquid CO_2

Critical Point Passage

When the purging is done, the chamber is cooled down to 0°C. The heat is then turned on and the sample is allowed to go critical (this takes from 12 – 20 minutes). Following critical point passage, the gaseous CO_2 is slowly bled off until the pressure is released. Samples are then removed from the dryer and the filters are mounted on stubs for coating.

2.8.2 Sputter Coating of the Samples

The samples are coated with gold in a Hummer VI sputter coater.

2.9 Sequencing

Samples were sent for sequencing to either Genewiz (South Plainfield, NJ) or MWG-Biotech (High Point, NC). Samples and primers were sent dissolved in water at room temperature. Because of the difficulty in sequencing *Chlamydomonas* genomic DNA both companies had trouble with our samples. However, Genewiz was able to supply us the useful partial sequences while MWG could not. Therefore, our TAIL-PCR sequencing was done at Genewiz.

2.10 Plasmid and BAC Isolation

All plasmid and BAC isolations were performed using Qiagen plasmid kits. Plasmid isolations were done according to the procedure described by the manufacturer. When isolating BACs, cultures were grown overnight at 37°C with shaking, and then 35 ug/ml spectinomycin was added to the culture to allow amplification of the BACs. Bacterial cultures were grown for another 7-8 hours with spectinomycin and the BACs were then isolated. Spectinomycin allows DNA replication but stops protein synthesis so it increases BAC concentration.

2.11 *Chlamydomonas* Cell Strains and Media

All strains of *Chlamydomonas reinhardtii* were maintained in our lab. CC strain numbers refer to the stock numbers from the *Chlamydomonas* culture collection (http://www.chlamy.org). The wild type strains used were CC-620 R3 MN mt^+ (wild type, mating type +) and CC-621 NO mt^- (wild type mating type -). The streptomycin resistant strain *C. reinhardtii* CC-275 (*sr-u-2-23,* mt^-) was used for mutagenesis. The wild type strain CC-124 (mt^+) was used for genetic analyses.

Stock solutions used to make media:

Beijerinck's solution

NH_4Cl	8.00 g
$CaCl_2.2H_2O$	1.00 g
$MgSO_4.7H_2O$	2.00 g
H_2O to 1000 ml	

Phosphate buffer (pH=7.0)

K_2HPO4	174.18 g
KH_2PO4	136.09 g
H_2O to 1000 ml	

Trace elements solution

EDTA (disodium salt)	50.0 g
$ZnSO_4.7H_2O$	22.0 g
H_3BO_3	11.4 g
$MnCl_2.4H_2O$	5.06 g
$FeSO_4.7H_2O$	4.99 g
$CoCl_2.6H_2O$	1.61 g
$CuSO_4.5H_2O$	1.57 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10 g
H_2O to 1000 ml	

Tris Acetate Phosphate (TAP) Growth Medium

Tris (hydroxymethyl aminomethane)	2.42 g
Beijerinck's solution	50 ml
Phosphate buffer	1.0 ml
Trace elements	1.0 ml
Glacial acetic acid	1.0 ml
H_2O to 1000 ml	

Beijerinck's solution for SEM-N

$CaCl_2.2H_2O$	1.0 g
$MgSO_4.7H_2O$	2.0 g
H_2O to 1000 ml	

Phosphate buffer for SEM-N

K_2HPO4	14.34 g
KH_2PO4	7.26 g
H_2O to 1000 ml	

Nitrogen-Free Medium (SEM-N)

Nitrogen-free Beijerinck's solution	50 ml
Phosphate buffer for SEM-N	75 ml
Trace elements solution	1.0 ml
H_2O to 1000ml	

2.12 SYBR Green I Staining

SYBR Green I (Invitrogen) was diluted 1:10 000 in TAE buffer. 2 parts of cells and 1 part of diluted dye were mixed and the cells were incubated in the dark at room temperature for 1 hour. After 1 hour, the cells were observed using a fluorescent microscope (Olympus BH2-RFCA).

CHAPTER 3

RESULTS

The goal of this study was to identify the genes that are involved in *mt*$^-$ gamete fusion in *Chlamydomonas.* To achieve this goal, in a forward genetics approach, nuclear mutants had to be generated by DNA insertional mutagenesis for later cloning and identification of the effected genes.

3.1 Mutants Generated

All nuclear mutants were generated by DNA insertional mutagenesis using the glassbead transformation technique (Kindle, 1990). The plasmid pSP124S was used to transform the host strain CC 275. Following transformation, fusion-defective mutants were selected by the streptomycin selection procedure (Forest and Togasaki, 1975). Because the streptomycin selection procedure used selects for all cells that cannot form zygotes, many non-agglutinating mutants were also isolated. Although non-agglutinating mutants were not the main concern of this study, some of them were kept for future studies. The list of the mutants generated is shown in Table 3.

All of the mutants in group 2 showed the fusion-defective phenotype; by phase contrast microscopy they were shown to form pairs and groups (indicating that they can adhere by their mating structures but are unable to fuse). Phase contrast micrographs and a scanning electron micrograph of this phenotype are shown in figure 28.

Table 3. List of mutants

	Mutant	cAMP Treatment	Pair Formation	Fusion	Date Isolated
Group 1. Non-agglutinating *mt*$^-$	7-26	Agglutinates with *mt*$^+$	ND	Fuses after cAMP treatment	2002
Group 2. Fusion-defective *mt*$^-$	cl-5	NC	Yes	No	2002
	cl-45 (isolated by Tammy La)				2001
	1-18*				8/2005
	1-23*				
	1-25*				
	1-35*				
	2-8**				9/2005
	2-29**				
	J1 (isolated by Jane Lam)				3/2006
Group 3. Mating Type Switch (agglutinates with mt-)	cl-1		ND	No	2002

* Four isolates from the same experiment
** Two isolates from the same experiment
NC: No change in phenotype

3.2 Determining the Number of Insertions in the Mutants

DNA insertional mutagenesis can result in transformants that randomly integrated more than one plasmid into the genome. Multiple mutations may occur if there are multiple insertion events within a cell, with only one of the insertions causing the fusion defective phenotype. Therefore, it is important to determine the number of insertions for fusion defective mutants by Southern blot.

Next, it is important to show the linkage of the insertion and the fusion defective phenotype. To show this, we would have to cross the mutant with a wild type *mt*$^+$. In

such crosses, one can observe the tight linkage of the phenotype and the insertion. Therefore, if the mutation results from the insertion, we would see fusion defective and Zeocin resistant progeny inherited together. However, because we are working with fusion defective mutants, and fusion is necessary to produce the zygotes that are analyzed in a genetic cross, we were not able to do the linkage analysis at this point.

3.2.1 Probes

The plasmid pSP124S which was used for DNA insertional mutagenesis was also used as the template for PCR to generate the *ble* probe. Figure 4A shows the sequence of the *ble* cassette. The shaded part is the piece amplified with primers bleforward and blereverse. These primers produce a 538 bp product. A probe for the *ori* region of pSP124S was also created with primers ORIAS and ORIS. The Southern blot result of this probe was not conclusive and is therefore not included here. Plasmid pSP109 was also labeled using the non-radioactive method.

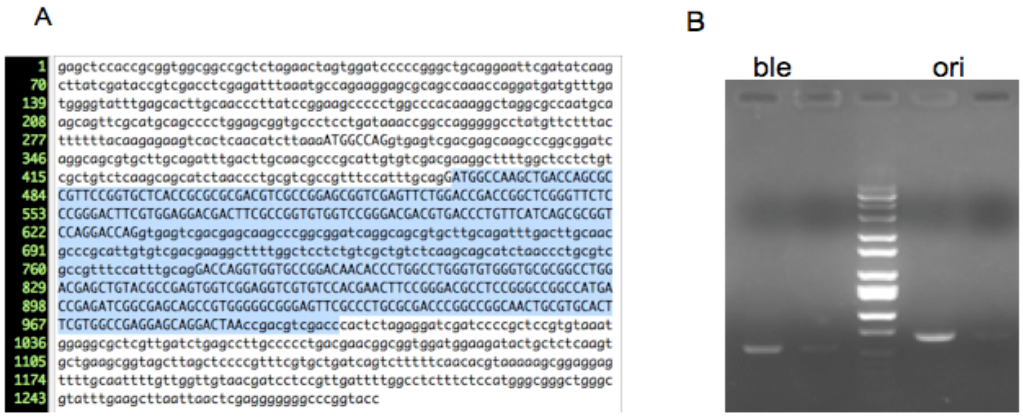


Figure 4. Probes Used for Southern Blots. A. The *ble* cassette. The shaded region shows the amplified part that was used for labeling. **B.** PCR products after gel extraction. The second lane for each probe shows the second elution. Only the first elutions were used for labeling. The *ble* product is 538 bp; the ori product is 659 bp.

3.2.2 Hybridization with the Probes

Figure 5 shows the Southern blot results. Figure 5A shows 2 different Southern blots. In the first panel, a photograph shows the southern blot for which genomic DNA was digested with Eco721. In the second panel a southern blot is shown where the genomic DNA was digested with KpnI or BamHI. The bands visible in the first panel are at higher positions, because Eco721 does not cut in pSP124S thus producing larger DNA fragments. In contrast, both KpnI and BamHI cut once within the plasmid pSP124S thus resulting in smaller DNA fragments.

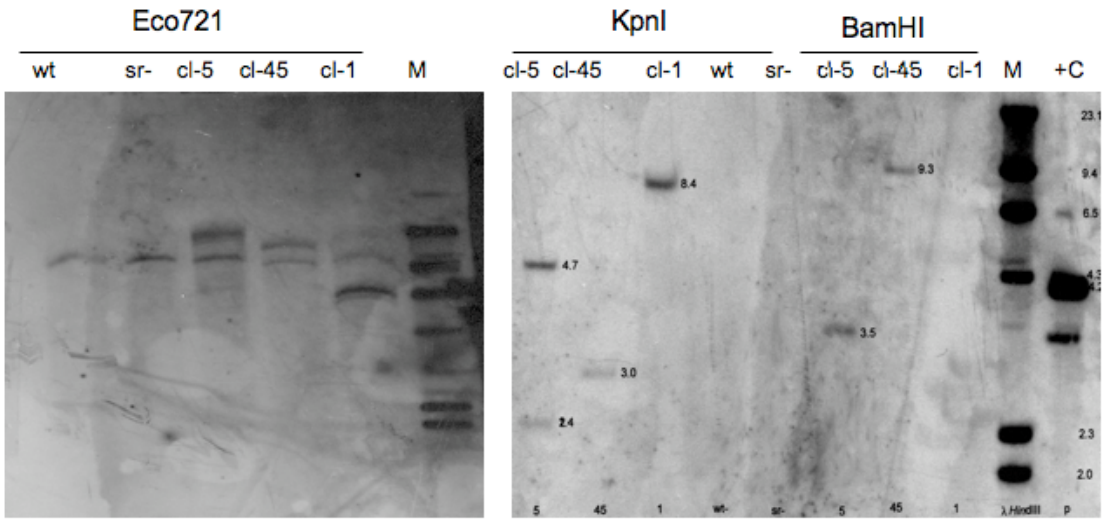
The first blot in figure 5A was done using the whole pSP109 as probe. For this labeling we expect to see a band for *wt* (CC-621 NO^-) and sr^-. We see a band around 10 kb in all strains. Besides this band, we see extra bands in the mutants; clone-5 has 2, clone-45 has 1 and clone-1 has one extra band. Clone-1 is mating type switch mutant which we did not work with further. In the second panel, we don't expect to see bands in the *wt* or sr^- strain, because the probe is only the *ble* sequence and it should not hybridize to anything in *wt* or sr^-. However, we should see the same number of extra bands (the insertion-specific bands) that we saw in panel one; that is what we see. Clone-5 has 2 bands, clone-45 has 1 band and clone-1 has 1 band. In these first southern blots it was noted that the upper band seen in clone-5 was very intense. However, when the Southern Blots were performed using a more sensitive radioactive labeling it became clear that this one intense band was in fact representing two bands (see the next paragraph).

In figure 5B the first lane labeled as sr^- (the strain used to create the mutants) is the negative control. As expected, we did not see any bands for sr^-. Clone-5 showed 3 bands, suggesting that it has 2 or 3 insertions, because the upper 2 bands might represent the same insertion possibly due to incomplete digestion. All the other mutants, except 1-35 and 2-8 showed 1 band, suggesting that all other mutants had 1 insertion. Although mutants 1-35 and 2-8 did not give any bands, they had the fusion defective phenotype. Mutant 2-8 was originally selected on the antibiotic Zeocin^{TM} (Invitrogen), but it does not grow on the antibiotic anymore indicating that the *ble* insertion might have been lost in this mutant. In contrast, mutant 1-35 grows on Zeocin, but the probe did not detect any bands. There have never been any reports of Zeocin resistant *Chlamydomonas* other than those produced by transformation with the *ble* plasmid, but we cannot rule out the

possibility that a mutagenesis event occurred during transformation resulting in Zeocin resistance. There is also a possibility of some structural change in the DNA of the insertion, making it undetectable by Southern blotting.

In summary, several mutants such as J1 had only on insert. The mutant clone-5 had several insertions.

A



B

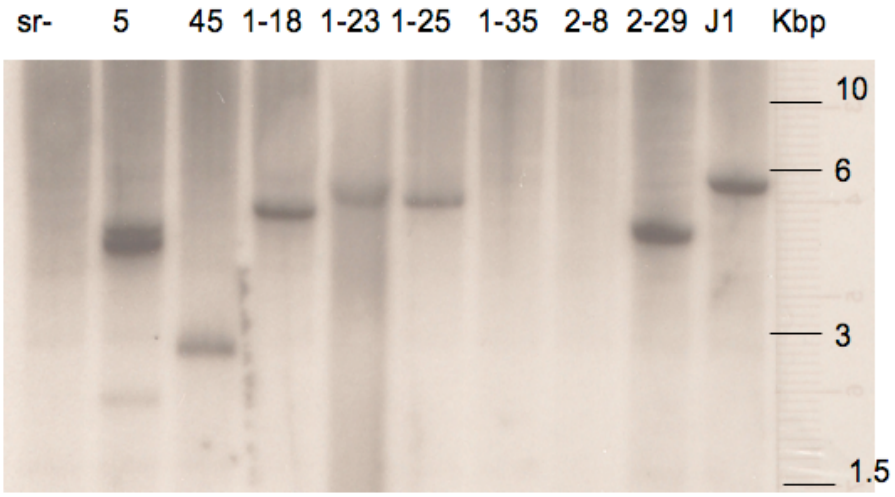


Figure 5. Southern blot results. A. Result of non-radioactive method. The probe for first blot was plasmid pSP109. pSP109 does not have the *rbcs2* intron but has the *rbcs2* promoter. Therefore *wt* and *sr*$^-$ both have a band. In the second blot, only the *ble* sequence was used to make the probe. Therefore *wt* and *sr*$^-$ do not produce any bands **B.** Result of the radioactive method. The *ble* sequence was radioactively labeled. As in the second panel of 5A, *sr*$^-$ does not have any bands as expected. M: DIG labeled DNA marker. +C: pSP124S.

3.3 Sequencing the Flanking Genomic Region of the Insertions

DNA insertional mutagenesis results in random insertions of the plasmid (in our case pSP124S) into the genome of *Chlamydomonas*. Insertions into the genome may disrupt genes or impact expression of genes. In order to find which gene or genes were disrupted by the insertion(s), it is necessary to determine the location of the insertion in the genome. To clone the regions flanking the inserts, the techniques of LMS-PCR (Ligation Mediated Suppression PCR) (Strauss et al., 2001) and plasmid rescue (Tam and Lefebvre, 1995) were attempted, but both techniques only produced vector sequence in those experiments (results are not shown here). Other laboratories had successfully used TAIL-PCR (Dent et al., 2005) and RESDA-PCR (Gonzalez-Ballester et al., 2005) to clone the flanking genomic region of the insertions in DNA insertional nuclear mutants of *C. reinhardtii*. Therefore, both techniques were applied here to clone the flanking regions for our mutants. In both techniques, primers designed for the insert (gene specific primers) and degenerate primers were used to amplify the flanking regions of the insertions.

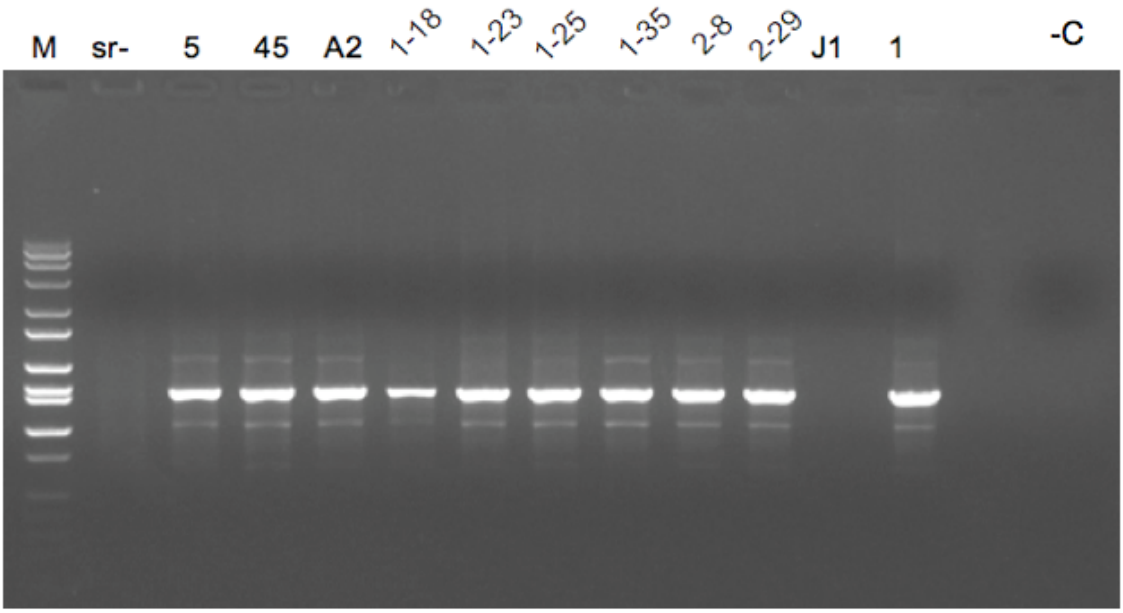
3.3.1 Sequencing the Flanking Region in Mutant Clone-5 by TAIL-PCR

3.3.1.1 Primary PCR

Figure 6A shows the primary PCR results using the gene specific primer Ble1 and the degenerate primer RD227. *sr*$^-$ was the strain used to create the mutants. We did not expect any product from the host strain *sr*$^-$, but in several cases some bands or a smear were found in primary PCR. This first primer pair did not produce any band for *sr*$^-$

although there was a very small amount of smear. We expect that the product formed in the mutants resulted from amplification of inserted pSP124S, because it was the same for all the mutants and was not present in *sr*$^{-}$. Mutant J1 did not give any product. The lane labeled as –C is the negative control lane, where no DNA was added to the PCR reaction. That the negative control –C had no product or smear, indicated that this primary PCR was good and we could continue to secondary PCR.

A



B

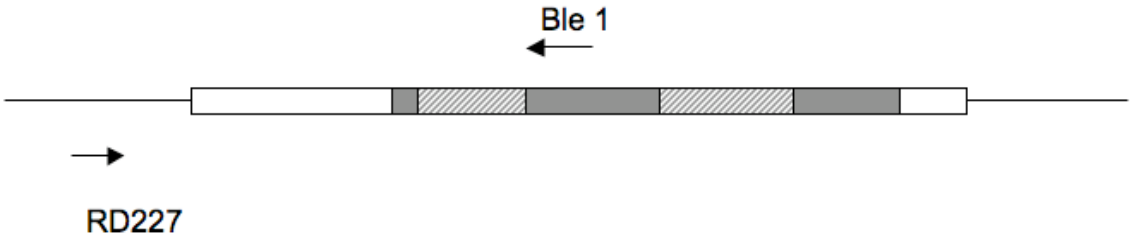
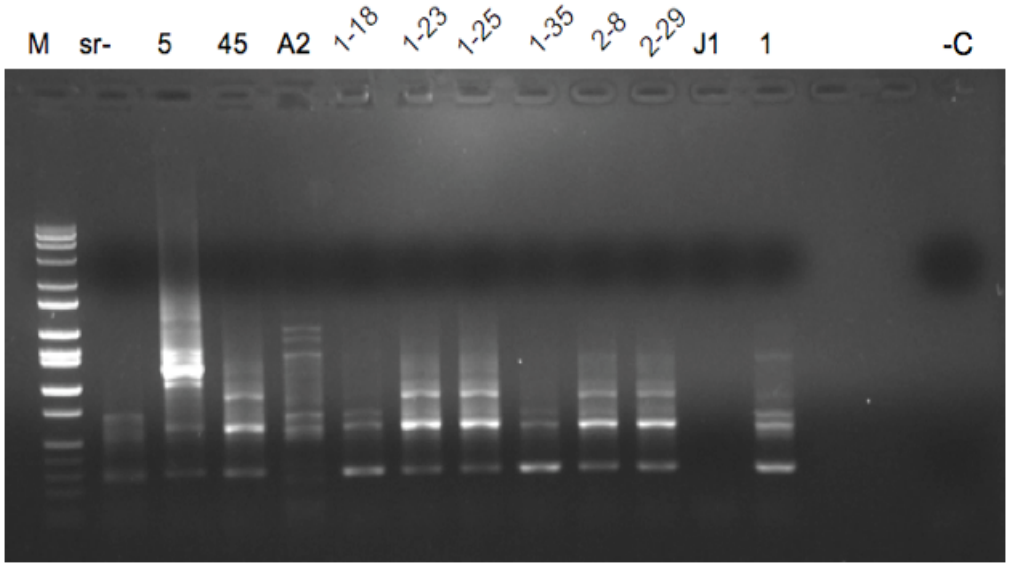


Figure 6: Primary PCR. A. Agarose gel for primary PCR products. **B.** Diagram showing the primer binding sites. Ble1 binds to the second exon of *ble*, RD227 is a degenerate primer that binds within the genome.

3.3.1.2 Secondary PCR

The secondary PCR was performed using the primary PCR products as templates. A nested gene specific primer, Ble2, that binds to the second exon of *ble* upstream of the primer used for primary PCR was paired with same degenerate primer, RD227. Figure 7 shows an example of the results obtained by secondary PCR. For Clone-5, a distinct, strong band became visible. For mutant J1 again no product appeared, but results were obtained by RESDA-PCR (see section 3.3.2). Again the negative control reaction shown in –C demonstrated that no contamination was present, therefore we continued to tertiary PCR.

A



B

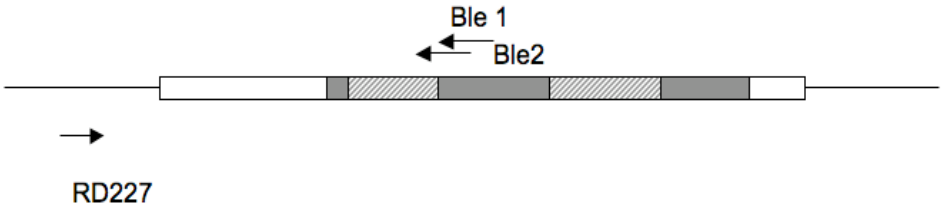


Figure 7. Secondary PCR. A. As expected, more specific products were formed in the secondary nested PCR. Clone-5 has a distinct band at ~1.2 kb. **B.** The relative position of the nested primer Ble2 to Ble1.

3.3.1.3 Tertiary PCR

The secondary PCR products were used as template for the tertiary nested PCR. This time, the gene specific primer BleC, that also binds to second exon of *ble* but closer to the 5'-end, and the same degenerate primer were used. Ideally tertiary PCR products should be very specific, but as shown in Figure 8a in many cases we did not get one distinct band. As Figure 8A shows, the distinct product of clone-5 became more concentrated in the tertiary PCR. But there was a smear above this band. In order to get a more specific PCR product, this band was gel extracted and used for further nested PCRs (Figure 8B). This band is a product produced by the primers BleC and RD227. BleC binds to the 305th base of pSP124S. RD223 and RD225 bind to the 121st and 75th base respectively. Therefore, each additional round of PCR resulted in the expected smaller sized products. We then sent the products of RD223 (1-1) and RD225 (1-2) to Genewiz for sequencing.

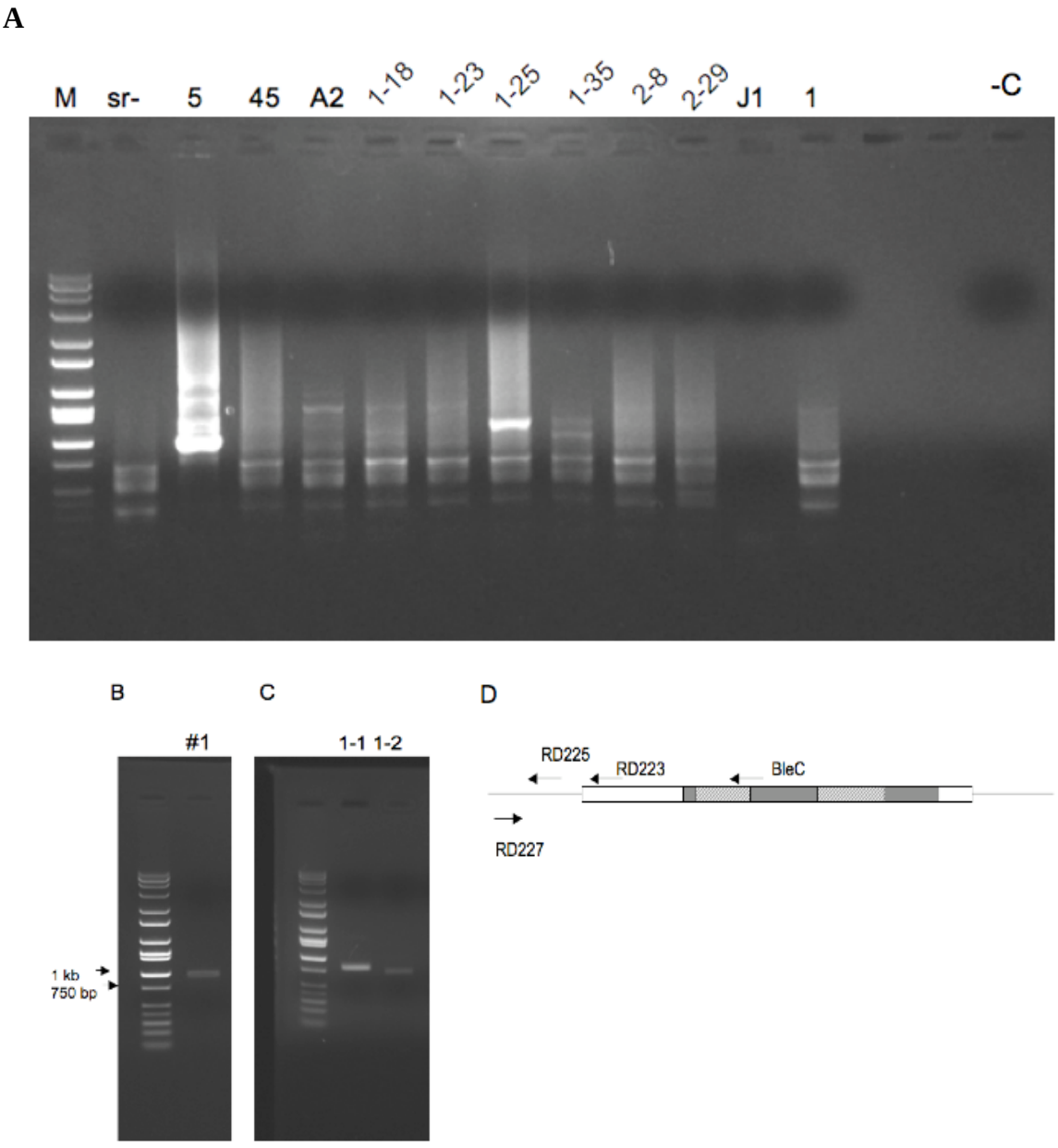


Figure 8. Tertiary PCR. A. Agarose gel of the tertiary PCR products. **B.** The band seen following gel extraction of the clone-5 tertiary PCR band. This band is around 1 kb. **C.** The gel extracted sample was used as a template for further nested PCRs. Product 1-1 is the product of primer RD223 and RD227. 1-2 is the product of RD225 and RD227. **D.** Position of primers on pSP124S.

3.3.1.4 Sequencing Results

When sample 1-1 was sequenced, we expected the sequence to contain the *rbcs2* promoter, followed by the pBluescript vector sequence, and then *Chlamydomonas* genomic sequences. When we blasted the sequence we received from Genewiz into the *Chlamydomonas* database, the sequence produced hits on multiple scaffolds as shown in Figure 9B. The longest matching genomic sequence was located on Scaffold 44.

A

```
ATAGGCCCCTGGCCGGTTTATCAGGAGGGCACCGCTCCAGGGGCTGCATGCGAACTGCTTGCCTTTNCGCCTAGCCTTTG
TGGGCCAGGGGGCTTCCGGATAAGGGTTGCAAGTGCTCAAATACCCCATCAAACATCATCCTGGTTTGGCTGCGCTCCTT
CTGGCATTTAAATCTCCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTC
GTTTTACAACGTCGCAGCATCATCTTGAAGCTGCTTGAACTTGACCAGTCCCGAGGACGAGTCATCCATCCTACCTAGCT
AGCGATAATAAGGCTTGCCAACCGCGAGCGAATTAACGACAAGCGGGGCTCATAACCTGTTGCGAAGTTGGTTCGGCAAG
CCTGAATTGCTAGCGGGTGCGTTACACACGATGCAGCTACGCTGCCTGGCACCTTCGGTAGTCTGGGTATATAATGCGCA
TGTGTAGTTGACAGCAACACGCTTGGTCGGGGCTTAAGACCTCCCGACGGTCCGTGAACCGACACAATGCCGTACTGGCA
GTCGGGGAGGCGCTACAGATGGGCAGACAAGGAGGCACTTCTGCCTGATCAGGGCAGGCGCCTCGCTAACTTCCCAGGGC
CCCAAGTTCGGACCGCAGCTACCACCTCACCGGCAGTGCTGCCCGATAACCCAAATTACCAACAGCTGGCACCATCTACT
GCTGCGCCTGTTGCGCCCGCTTGGCCTNGGTCCGCCGCGCCCCCGCGTCNAGACCTCTTGCGCAGCCGCACCGCCGCAGG
GTCACCTCTATGACTCGTCTNGGCANATACNTGCAGNGGNCGGGN
```

B

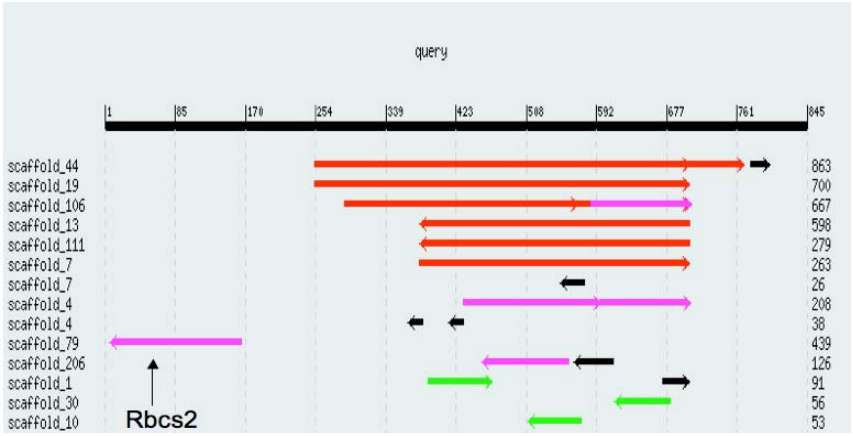


Figure 9. Sequencing Result of Sample 1-1. A. Sequence of the product. **B.** ChlamyDB blast result.

In addition, sample 1-2 was sequenced with primer RD225. From this PCR product we expected to obtain a short pBluescript vector sequence followed by *Chlamydomonas* genome sequence. The results of this sequencing reaction are shown in Figure 10A.

A

```
GGGGGGCCGGTCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAANGTCGCAGCATCATC
TTGAAGCTGCTTGAACTTGACCAGTCCCGAGGACGAGTCATCCATCCTACCTAGCTAGCGATAATAAGGCTTGCCAACCG
CGAGCGAATTAACGACAAGCGGGGCTCATAACCTGTTGCGAAGTTGGTTCGGCAAGCCTGAATTGCTAGCGGGTGCGTTA
CACACGATGCAGCTACGCTGCCTGGCACCTTCGGTAGTCTGGGTATATAATGCGCATGTGTAGTTGACAGCAACACGCTT
GGTCGGGGCTTAAGACCTCCCGACGGTCCGTGAACCGACACAATGCCGTACTGGCAGTCGGGGAGGCGCTACAGATGGGC
AGACAAGGAGGCACTTCTGCCTGATCAGGGCAGGCGCCTCGCTAACTTCCCAGGGCCCCAAGTTCGGACCGCAGCTACCA
CCTCACCGGCAGTGCTGCCCGATAACCCAAATTACCAACAGCTGGCACCATCTACTGCTGCGCCTGTTGCGCCCGCTTGG
CCTTGGTCCGCCGCGCCCCCGCGTCCAGGACCTCCTTGCGCAGCCGCACCGCCGCAGGGGTCACCTCTATGAGCTCGTCC
TGGGCAACATACCTGCAGGCGGGCCGGGGCCGGCGGGTTTATGTTGCAGTCAGTAAGCACGGGGCCCTCCTTCTCGNTNN
NNNNNNANNAAAAAAAANNGGNNGGNNNNNNNNNGCNGNNNNNNNNNNAAAAAANNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

B

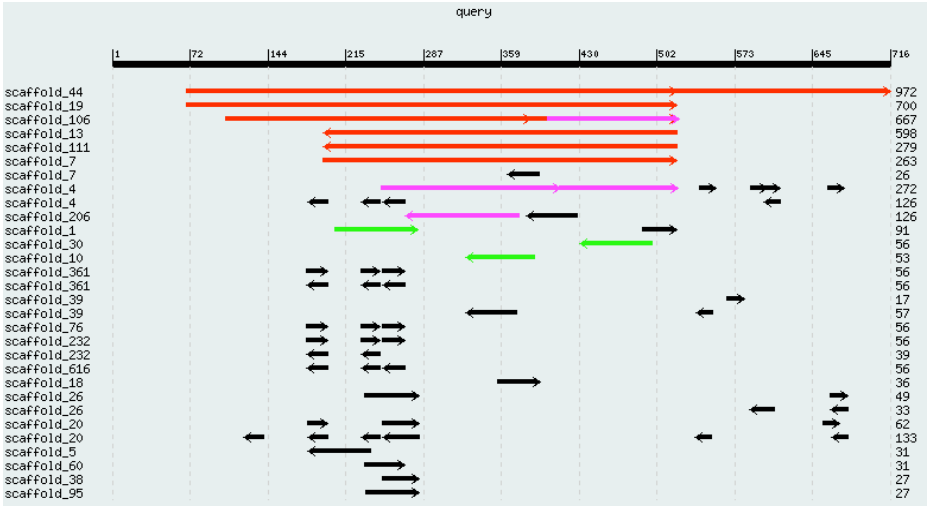


Figure 10. Sequencing results of sample 1-2. A. Sequencing result. **B.** ChlamyDB genome browser showing the blast result.

When the obtained sequence was used for a blast analysis using the *Chlamydomonas* genome database, we saw a result that was similar to the one obtained from the blast with the sequence from sample 1-1 (Figure 10B). Again the longest identical genomic sequence was located on Scaffold 44, indicating that the plasmid insertion happened in this region of the genome. However, for this reaction, scaffold 79 no longer showed up, indicating that for this sample, the primers were indeed beyond the region with the *rbcs2* promoter insert and now began in the pBluescript vector sequence and then continued into the *Chlamydomonas* genome.

In summary, both results indicated that clone-5 carried an insertion in scaffold 44 of the *C. reinhardtii* genome.

3.3.1.5 Walking into the Genome from the Insertion

In order to prove that our TAIL-PCR sequencing had correctly identified the genomic region of Scaffold 44 as one insertion site and to test for intactness of the genomic region around the insertion site, new primers were designed that were specific for adjacent sites of the genomic region on Scaffold 44. PCR was then performed with these genome specific primers and the *ble* primers. In addition to the mutant cl-5, some other mutants were also tested for possible insertions in the region of Scaffold 44. We should not get any product from the other mutants, unless they also have an insertion in very close proximity to the insertion in Scaffold 44 of cl-5. Primers RD233 and NSG3R2 should produce a 759 bp product. Figure 11A shows this expected product from the cl-5 PCR, but not from the other mutants tested, cl-45 and 1-25. This result indicated that mutants cl-45 and l-25 did not contain an insertion in that position of Scaffold 44. In figure 11B, the left side of the gel shows the product of PCR with RD223 in pair with NSG3R4. This reaction gave a product of the expected size (1848 bp). The right side of the gel shows the result of PCR performed with BleC and NSG3R2. The product size is 942 bp, as expected. These results confirmed the results of the TAIL-PCR by demonstrating that one insertion of cl-5 occurred in Scaffold 44.

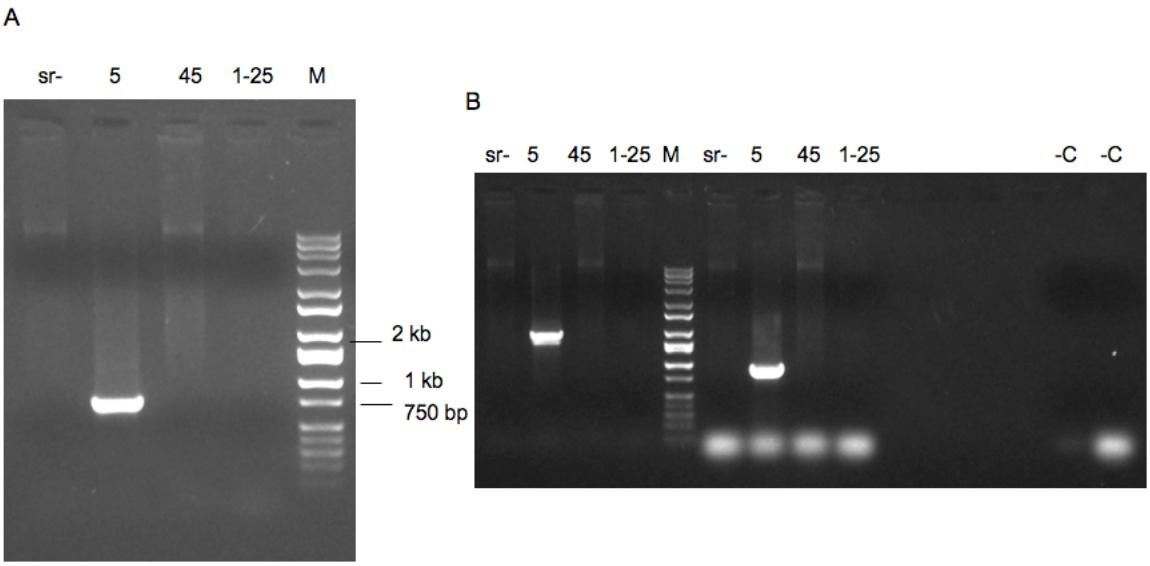


Figure 11. Genome walking from the insertion in clone-5. A. PCR with RD223 and NSG3R2 gave a 759 bp product. **B.** The left side shows results of PCR with RD223 and NSG3R4. The product is 1848 bp. The right side shows results of PCR with BleC and NSG3R2. The product is 942 bp. The last two lanes are negative controls (–C) from the first and second PCRs respectively.

3.3.1.6 The Insertion Site for Clone-5

The plasmid insertion in clone-5 occurred in scaffold-44; as concluded from the sequencing result of sample 1-2, the exact base position is 523923. Figure 12 shows the genome browser image of this region. The black down arrow shows the insertion site. Version 3 of the *Chlamydomonas* genome did not have any gene models at this position, but there are 2 gene models upstream and downstream of the insertion. Gene model C_440054 (blue, base position 504940-517323) was annotated as having a carboxypeptidase motif. Gene model 44.48.1 (red, base position 524387-529724) was annotated as having homologies to both a GTP binding protein and an elongation factor.

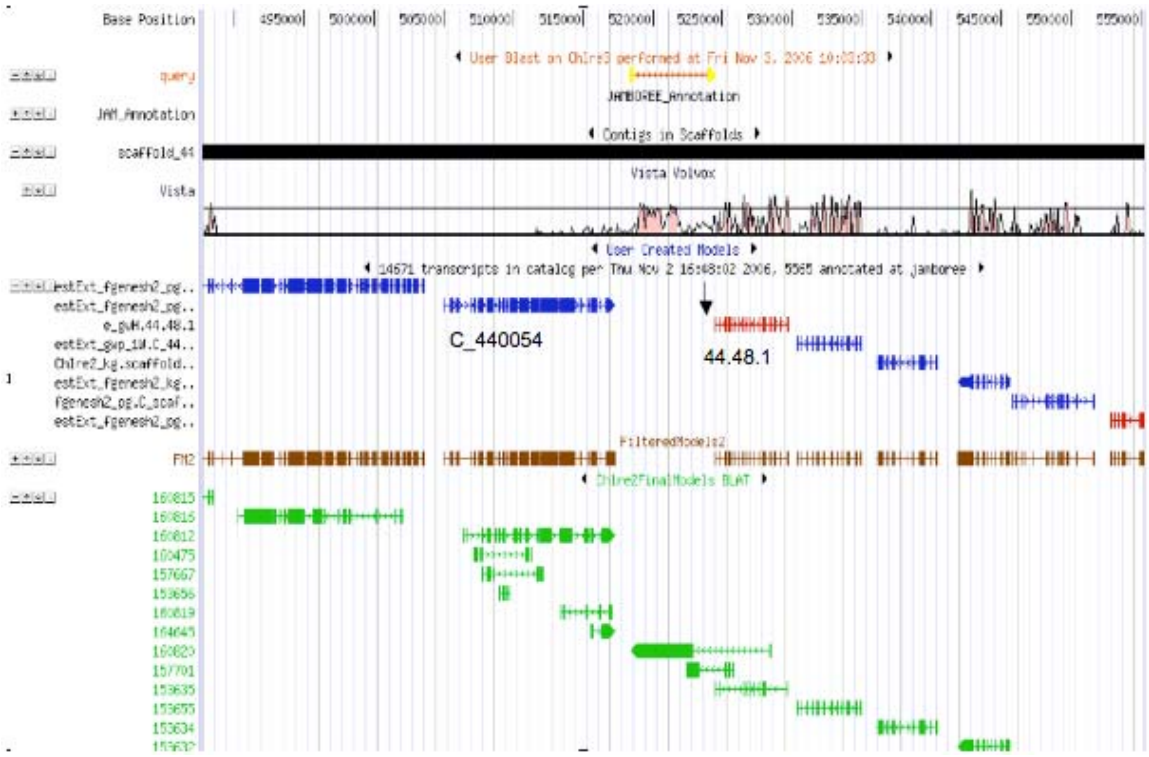


Figure 12. Region of insertion in clone-5. Genome browser image of the region of the insertion in scaffold-44. The black down arrow shows the insertion site. Gene model left to the arrow is C_440054 and gene model on the right of the arrow is 44.48.1.

3.3.2 Sequencing the Flanking Region in J1 by RESDA-PCR

According to our Southern Blot results, the mutant J1 had only one insertion which may be responsible for the mutant phenotype. Cloning of the flanking regions of that insert should allow us to determine the gene(s) that are disrupted by the insertion which we hope to prove caused the fusion defect.

3.3.2.1 Primary PCR

Genomic DNA of mutant J1 was used for cloning of the flanking regions by RESDA-PCR. Figure 13 shows the primary PCR results for multiple mutants, including J1 using the gene specific primer RD223 and the degenerate primer Eco721. All samples show smears, which usually occurs for primary PCR.

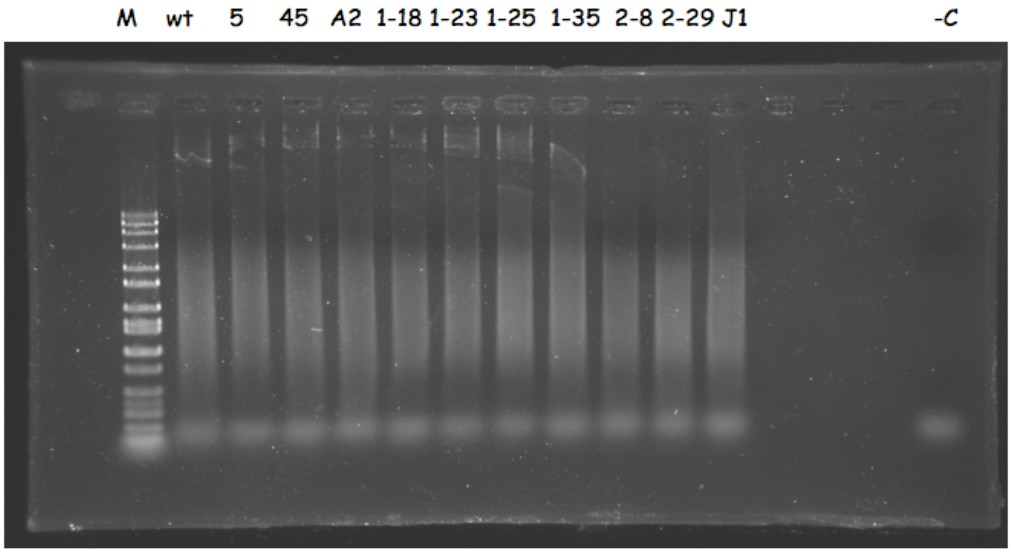


Figure 13. Primary RESDA-PCR results.

3.3.2.2 Secondary PCR

Secondary PCR was performed using the primary PCR products as the template. In RESDA-PCR, the insert specific and degenerate primers used in the secondary PCR are both nested, to get more specific products. Primer RD224 and Q0 (the nested primers for degenerate primers used in primary PCR) were used. The priming site for RD224 is still in the *rbcs2* promoter, but closer to 5'-end. The result of the secondary PCR is shown in figure 14. While there are differences between samples, no specific products were identified for any of the mutants.

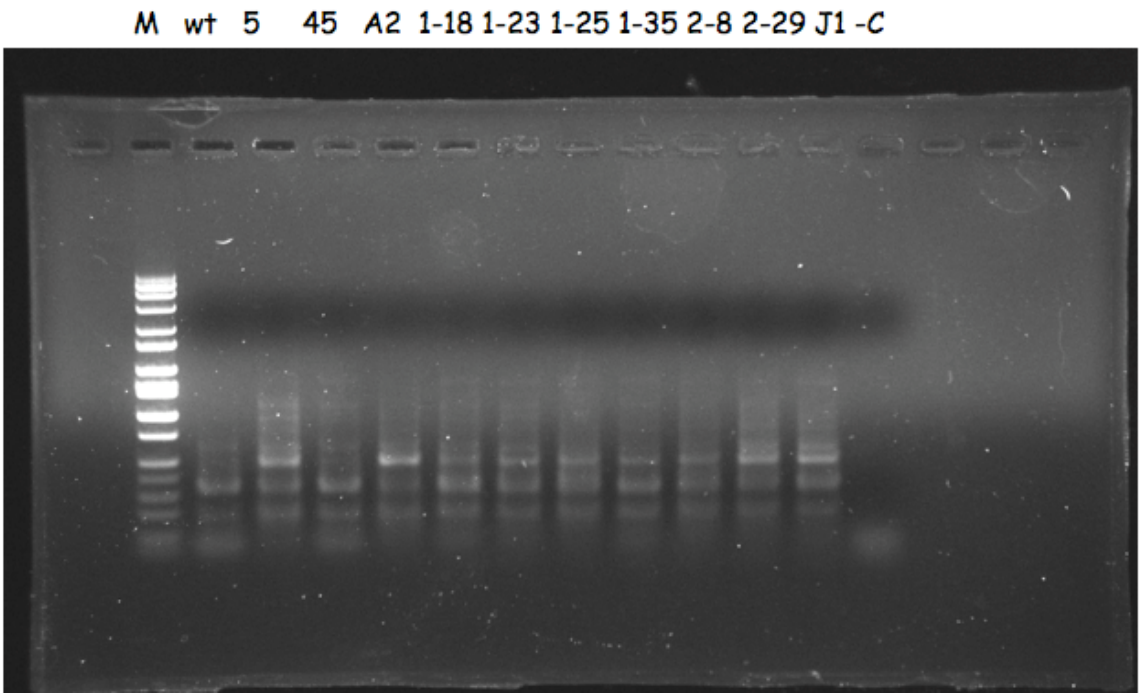


Figure 14. Secondary RESDA-PCR results.

3.3.2.3 Tertiary PCR

The secondary PCR products were used as templates for the tertiary nested PCR. The primers were RD225 and Q0. Compared to the tertiary TAIL-PCR results, the tertiary RESDA-PCR gave more specific products. This could be due to use of nested gene specific primers and primer Q0, which is a nested primer for the degenerate primer used in primary PCR. This time a very specific product was identified for the mutant J1. This band from mutant J1 was gel extracted and was sent, along with the primer RD225, to Genewiz for sequencing.

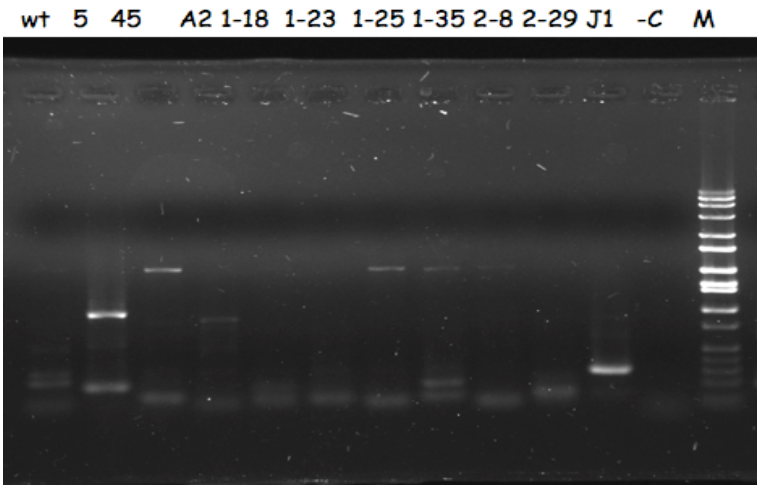


Figure 15. Tertiary RESDA-PCR results.

3.3.2.4 Result of Sequencing

The product obtained in this tertiary PCR of mutant J1 was around 292 bp long (Figure 16A). Since we sequenced this with the RD225 primer, we expect to see some vector sequence followed by *Chlamydomonas* genome sequence. This expected result is reflected in figure 16B showing the result of a BLAST into the *Chlamydomonas* genome. When blasted, this sequence had only one match in *the Chlamydomonas* genome, which was in scaffold 37.

A

GGACCTTCGCGGAAAGACGTGTACAATGACAACTGGCTTGACCTCCTGTTCA
TCAAGCTCTACTCTANGAAGATGGCGGACTGCCTCCCAGCTAGCCAGGGTAC
GACCAGGCGGTATGACCGAGCAGGCCGGTCGTGGGGGCAGCGTTTGGACGTG
TGGATTGAGCGCACCGGCAGACCTGTTCACGCGCGCTCTTCGTCTCACTTCGT
TGTGCTCTATCGCATAGAGCACTGGGCACAGTGCTCCCCCCCGTCACTCTGCC
TCACTGGAAATTCTAATNAAGTTTNNACAC

B

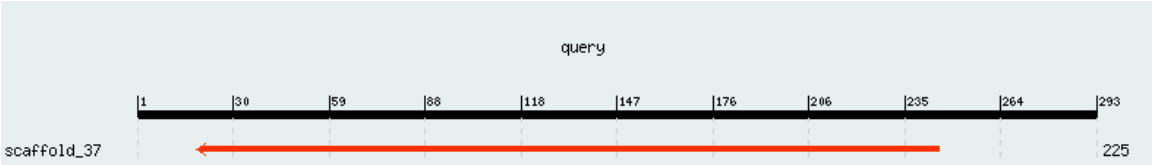


Figure 16. Sequencing results for J1. A. The sequencing result. **B.** Blast result from the ChlamyDB. The sequence had only one match in the ChlamyDB, in scaffold-37.

3.3.2.5 Walking into Genome from the Insertion

To confirm that the plasmid pSP124S inserted into the genomic region of Scaffold 37, primers were designed for scaffold-37 and PCR was performed with primers RD225 and J1-F. Figure 17 shows that PCR of genomic DNA from mutant J1 generated a product of the expected size of 987 bp.

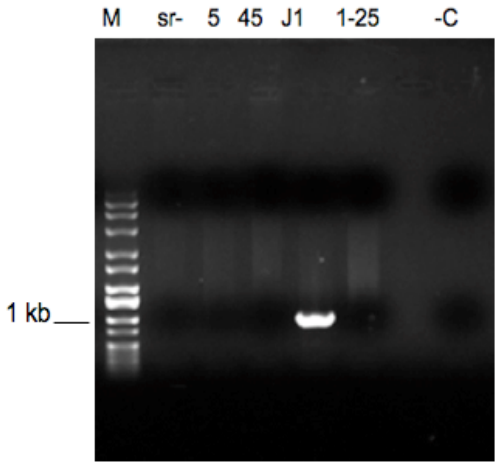


Figure 17. Genome walking for J1. Only J1 genomic DNA gave a PCR product as expected.

3.3.2.6 The Insertion Site for J1

The insertion in J1 occurred in scaffold-37 with the insertion disrupting gene model C_370109. C_370109 is annotated as an expressed protein in *Volvox* and *Arabidopsis* but does not have any known function in these organisms.

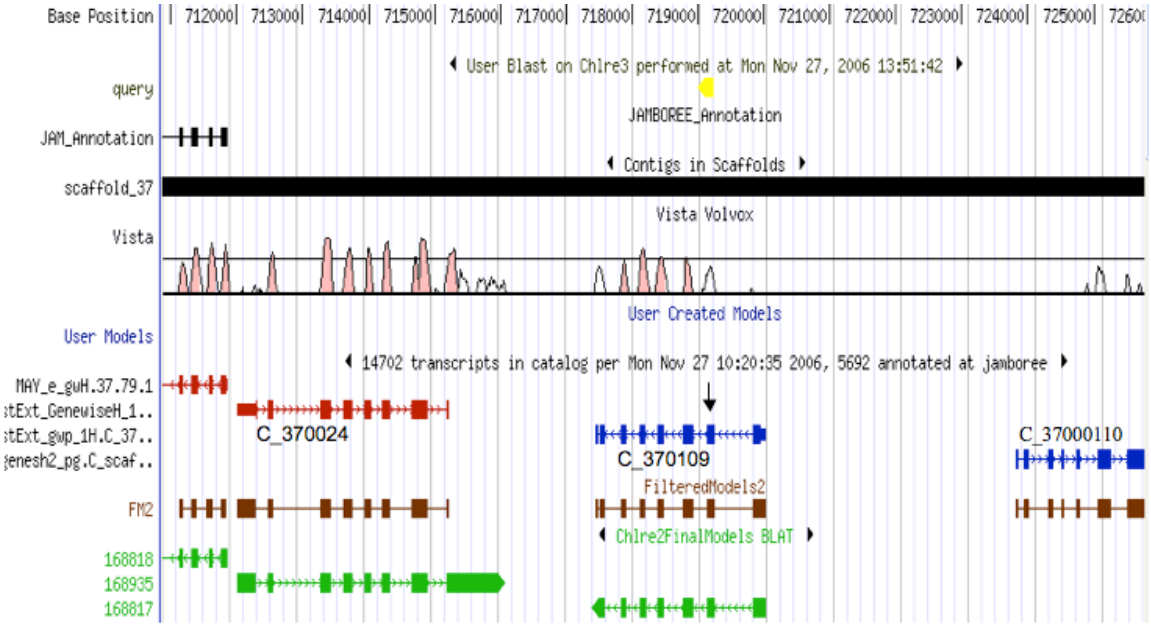
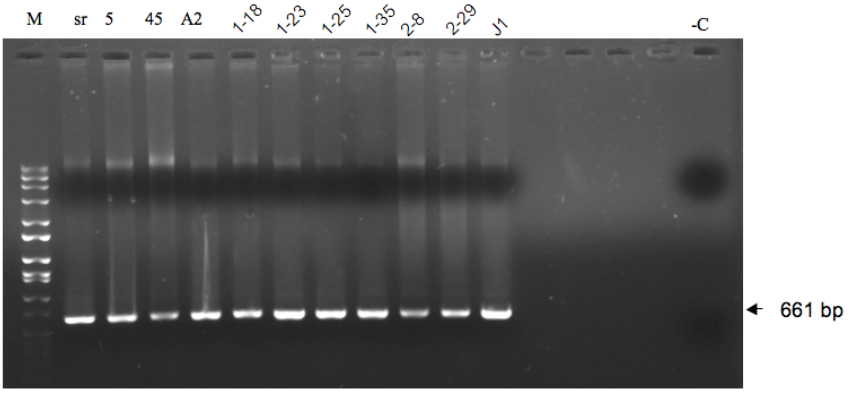


Figure 18. Region of insertion in J1. The down arrow shows the insertion site in gene model C_370109.

3.3.2.7 Determination if there is a Possible Deletion in the Insertion Region

Previously it was shown that DNA insertional mutagenesis can result in large deletions when the insertion happens (Cenkci et al., 2003). To test for intactness of the genomic region at the insertion site in Scaffold 37, we performed diagnostic PCR for regions upstream and downstream from the insertion site. We know that the insertion happened in position 719224 bp of scaffold 37 and we were able to sequence the 225 bp downstream of this insertion point. Figure 19A shows the PCR result from downstream of the insertion using primers J1-F and J1-R. These primers amplify a 661 bp product in the scaffold (position 718330 and 718991). All the mutants, including J1 gave the right size product, indicating that the region upstream of the insertion site was intact. Figure 19B shows the PCR result from downstream of the insertion site. PCR was performed with primers j1-f2 and j1-r2. Again all mutants gave the same size product (1782 bp); therefore we can conclude that the downstream region adjacent to the insertion site was intact. We did PCR further downstream with primers j1-f3 and j1-r3. The result of this PCR is not shown here, but we again got product for all the mutants. These primers amplify a 1362 bp region. The priming site of these primers is not in gene model C_370109. It is in the downstream gene model C_37000110.

A



B

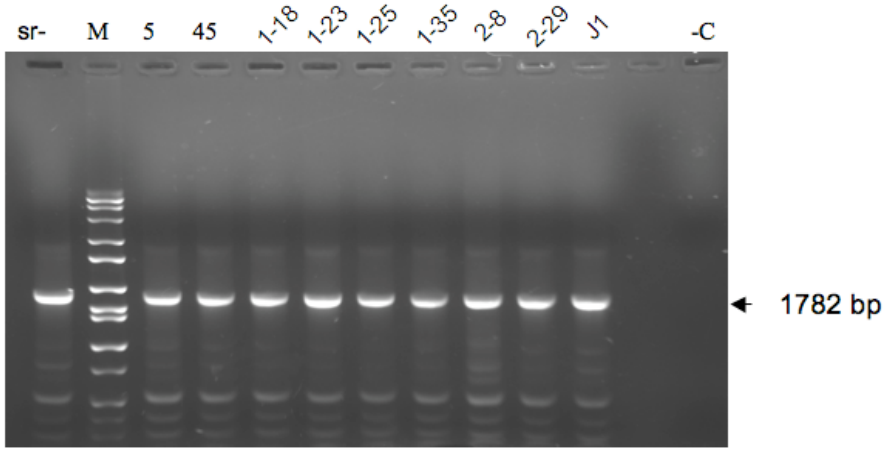


Figure 19. Determining if there is a deletion in the J1 insertion site. A. PCR for the region upstream of the insertion gave product for all of the mutants, including J1, indicating that there is no deletion. **B.** The region downstream of the insertion is also intact.

3.4 BAC Modification

Chlamydomonas BACs do not have any selectable marker gene for our strain (CC 275). In order to be able to select cells that have been transformed with the BAC, we wanted to insert a selectable marker into the BACs.

3.4.1 Creation of the Insert (Fusing the Hygromycin and Kanamycin Cassettes)

First, the Hygromycin cassette was ligated to pBluescript SK-. This plasmid was named pHYg3MA and it is 4.7 kb (Figure 20A and B).

Next, the gene conferring kanamycin resistance was amplified from two different sources: The first was the Tn5-neo cassette which was included in the BAC modification kit. This cassette was amplified with primers Tn5-neo-f and Tn5-neo-r. This product is 947 bp. The PCR product was gel extracted and ligated into pGEM-T Easy vector using 3:1 insert to vector ratio.

The second kanamycin cassette was amplified from pACYC177 (New England Biolabs). The PCR products were gel extracted and ligated into pGEMT-Easy vector. Figure 20A shows these two ligation products after NotI digestion. NotI cuts on both sides of the MCS of pGEM-T Easy, resulting in excision of the insert. The kanamycin cassette amplified from pACYC177 is 1082 bp, whereas the neomycin cassette amplified from the kit's template is 947 bp. The difference in length can be seen in the gel (Figure 20A), the Tn5-neo product is slightly smaller. After Not I digestion, the excised cassettes were gel purified and ligated into NotI digested, gel purified pHyg3MA. NotI linearizes the pHyg3MA (Figure 20A). Even thought we generated 2 vectors, we decided to continue with the Tn5-neo construct, which was named pHyg3MA-Tn5neo (the reason is explained in the next section).

pHyg3MA-Tn5neo should be 5.7 kb. Figure 20B shows the SspI digestion of both vectors. SspI cuts twice in pBluescript but not in the hygromycin or neomycin cassettes. The excised vector piece is 553 bp. pHyg3MA-Tn5neo is 1 kb bigger then the pHyg3MA because of the neomycin cassette insertion (Figure 20B).

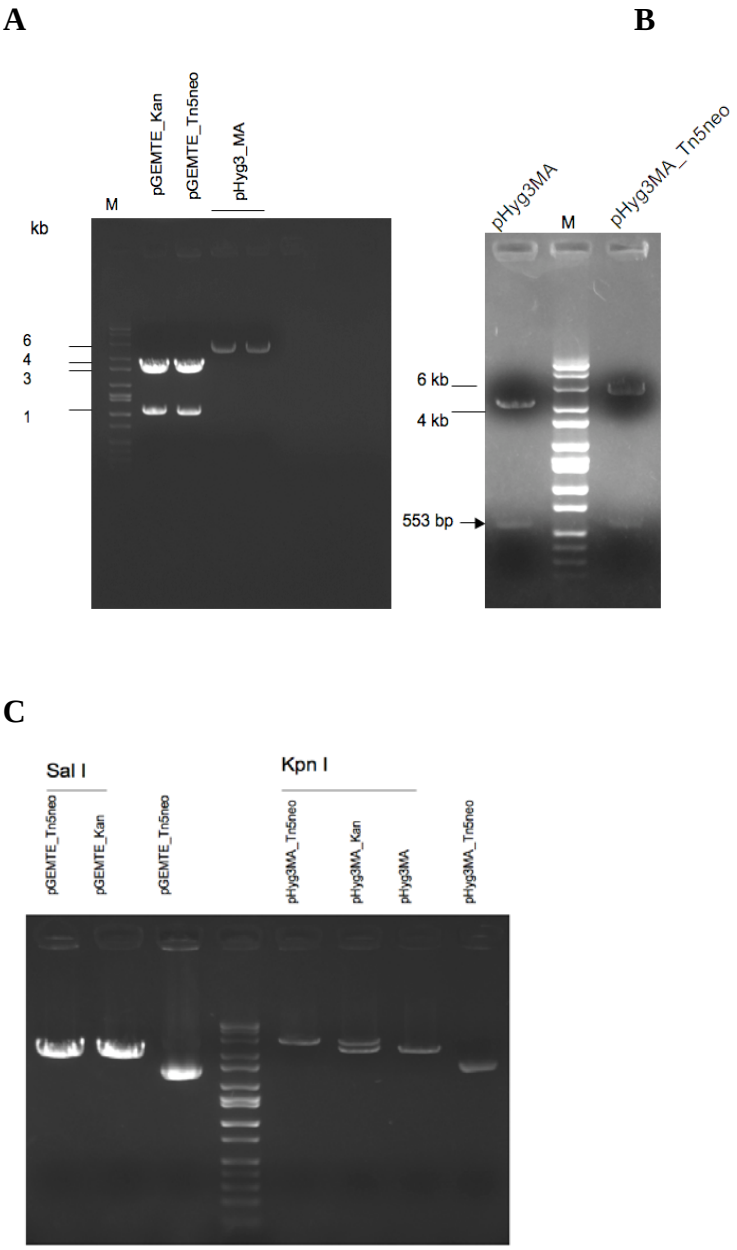


Figure 20. Ligation of Hygromycin and Kanamycin cassettes for BAC recombination. A. The kanamycin gene amplified from pACYC177 and the Tn5-neo cassette amplified from the kit's cassettes were ligated to pGEM-T Easy vector. Not I digestion of vectors. **B.** Ligation of the kanamycin cassette next to the hygromycin cassette in pHyg3MA. SspI digestion. **C.** All the vectors created for BAC recombination. Undigested pGEMT-Tn5neo in the first and pHyg3MA-Tn5neo in the second panel were also loaded onto gel to make a comparison with the digested plasmids.

3.4.1.1 Amplification of the Fused Hygromycin and Kanamycin Cassettes

pHyg3MA-Tn5neo and pHyg3MA-Kan were used to amplify the fused hygromycin and kanamycin cassettes. Amplification with homology arm primers produced extremely low amounts of product. To solve this problem, I first amplified the cassettes with normal primers (Figure 21A, T7 BGalf section), then used this product as template to create homology arms with HA primers (Figure 21A, HA primers section). Even then, the product of HA primers was small, but sufficient to be used for recombination. As shown in Figure 21A, pHyg3MA-Tn5neo gave one product of 3 kb as expected, but the pHyg3MA-Kan vector gave more then 1 product. One of them was as in pHy3MA, which is expected to be 1.7 kb, because pHy3g3MA has only the hygromycin cassette in MCS. This told us that bacterial clone had two versions of the plasmid: One was the recircularized pHyg3MA (which is possible because NotI produces sticky ends), while the other plasmid had the ligated kanamycin cassette. Figure 20C shows this clearly; pHy3MA-Kan linearized with KpnI has two plasmids, whereas, pHyg3MA-Tn5neo has only one product. Therefore, we decided to use pHyg3MA-Tn5neo to amplify the fusion cassettes. Previous PCR reactions were carried out with the Epicentre polymerase, which gave a lot of product; however this polymerase mix also contains non-proof reading polymerases. In homologous recombination, the piece to be inserted has to have 100% homology to the target. Therefore, we needed a polymerase with high accuracy. We found Finzyme's Phusion Polymerase (New England Biolabs) to be the highest fidelity enzyme in the market. However, as Figure 21B shows, this enzyme did not produce as much product as the Epicentre enzyme mix did. Multiple PCR reactions had to be performed and the DNA had to be concentrated by gel extraction to get enough to use in recombination.

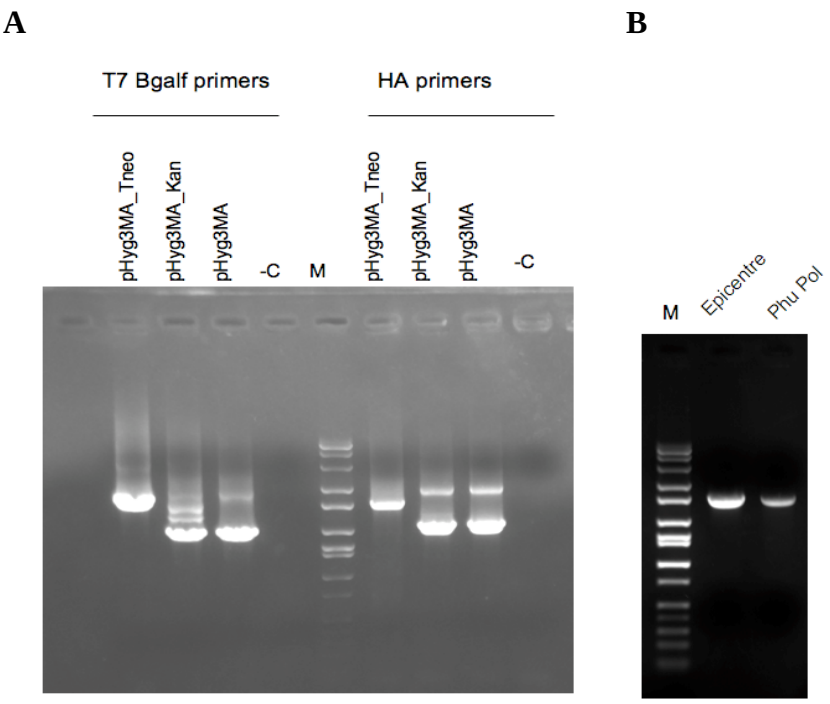


Figure 21. Generation of an insert with homology arms for recombination. A. Amplification of the fused hygromycin and kanamycin cassettes. The first panel was done with primers without homology arms to generate a template for the second round of PCR with homology arm primers. The second panel is the reamplification of the template generated in the first panel with homology arms. **B.** Gel purification of the insert. Comparison of Epicentre and Finnzyme polymerases. The Phu polymerase product was used for recombination.

3.4.2 Recombination

By fusing a prokaryotic and a eukaryotic expression cassette, and then using this fused cassette to create an insert for recombination, we were able to select correctly recombined clones in one step recombination: Otherwise we would have needed to do two recombination events which would be more time consuming.

We were able to get sufficient amount of correctly recombined bacterial cells in one experiment (Figure 22).

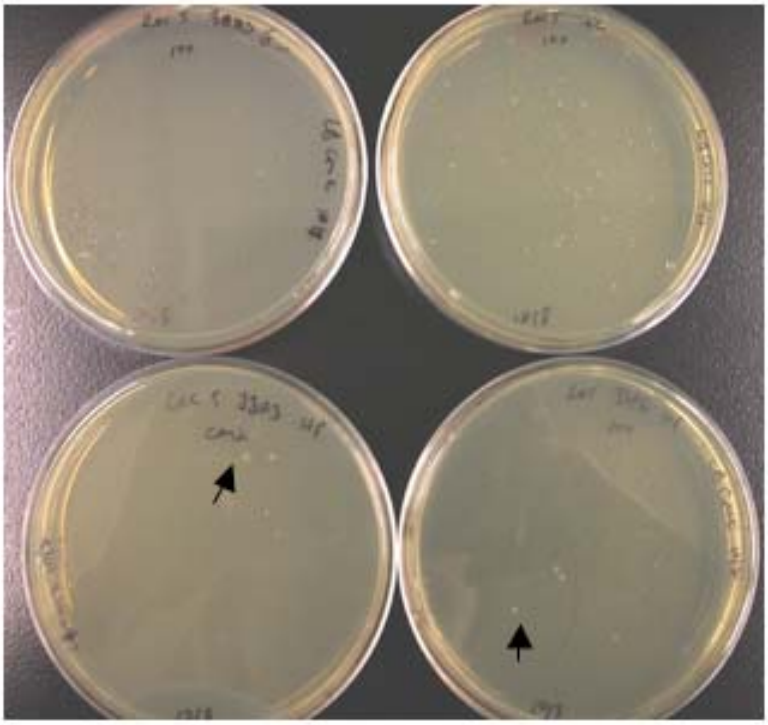


Figure 22. Result of recombination. The plate in the upper left shows uninduced recombination for BAC 33A3 (-C uninduced). There are no colonies, as expected (it is normal to have very small colonies but they are not correctly recombined. The purpose of doing uninduced recombination is to compare the size of colonies in –C and in experimentals. The correctly recombined colonies are much larger then the colonies growing on –C plate). The upper right plate is +C. The lower left shows recombination of BAC 33A3. The lower right plate shows recombination of BAC 37P6. The arrows are pointing to recombinant colonies which now show kanamycin resistance.

3.4.3 Verification of Successful Recombination by PCR

After recombination, bacterial cells are plated on LB plates containing chloramphenicol and kanamycin to select for recombinants. However having the insertion cassette inserted in the BAC does not always mean the insertion occurred where we wanted it to be. In some cases, the bacterial cell receives the insertion, becomes resistant to kanamycin, but the insertion happens in some other place. This type of recombination is called secondary recombination. We cannot use secondary recombinants in future complementation experiment. Therefore we need to verify recombination in the resistant colonies we get after the recombination event.

The diagram in Figure 23 shows the position of the *nrt2* gene in pBACmn. The *nrt2* gene was inserted into the unique XhoI site of pBeloBAC11 to create pBACmn. pBACmn was then used to create the *Chlamydomonas* BAC library as follows. The *Chlamydomonas* genome was partially digested with HindIII, size fractionated and ligated into unique HindIII site of pBACmn. The ligation was used to transform *E.coli,* and colonies were selected by color complementation.

By inserting our fused cassettes, we replaced a 3175 bp region from the *nrt2* gene. The reason we chose to delete this region is because the *nrt2* gene is not useful in our complementation experiment. This 3175 bp region was replaced with our 3103 bp insert (the fused hygromycin and kanamycin cassettes and 50 bp homology arms at each end).

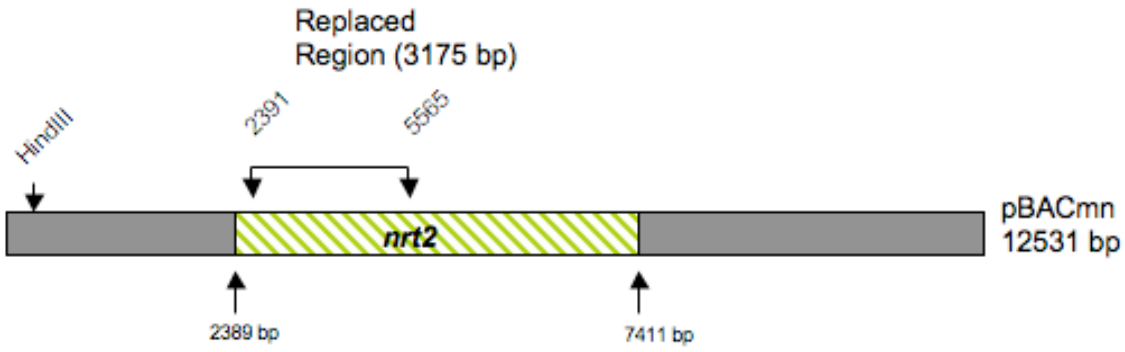
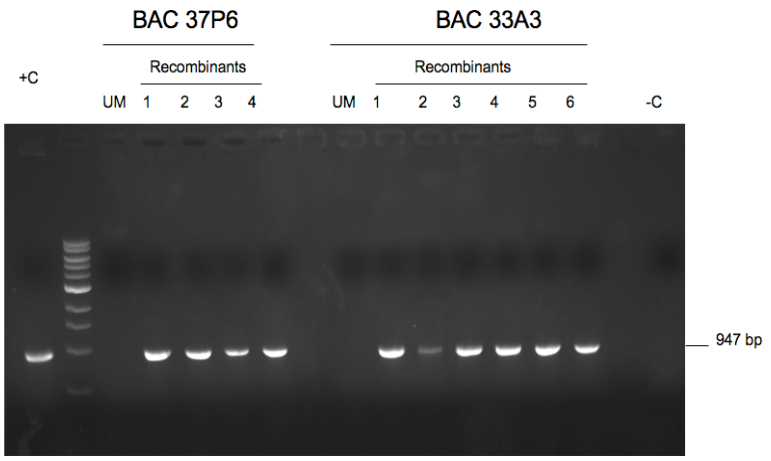


Figure 23. Schematic representation of recombination. The diagram is not drawn to scale.

3.4.3.1 Presence of Hygromycin and Kanamycin Cassettes in the Modified BACs

We did PCR to show the presence of the hygromycin cassette and the kanamycin cassette in the recombinant colonies, using the original unmodified BACs as a negative control. Only the modified BACs should have a PCR product. Figure 24A and B show the result of PCR for the kanamycin and hygromycin cassettes respectively. As expected, only the modified BACs have product in both reactions. Primers Tn5-neo-f and Tn5-neo-r were used to amplify Tn5-neomycin cassette. Primers Hyg3-f2 and Hyg3-r2 were used to amplify the hygromycin cassette.

A



B

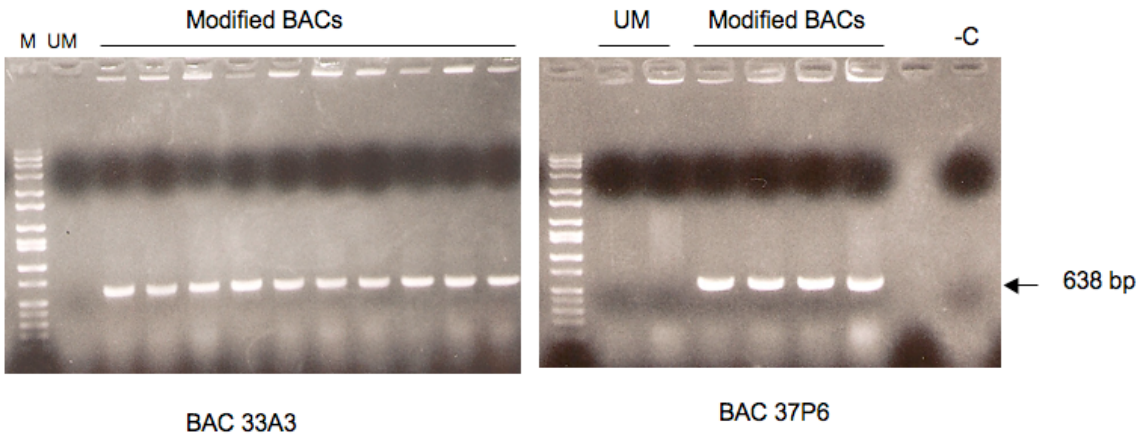


Figure 24. Presence of Kanamycin and Hygromycin cassettes in the recombinant BACs. A. PCR for the Tn5-neo cassette. Only the modified BACs have a product which is 947 bp. **B.** PCR for the hygromycin cassette. Again only the modified BACs have a product which is 638 bp.
+C: BAC control. This experiment is done with a control BAC and an insertion cassette (Tn5-neo cassette) which was included in the kit as a positive control for the experiment.
–C: negative control for the PCR.

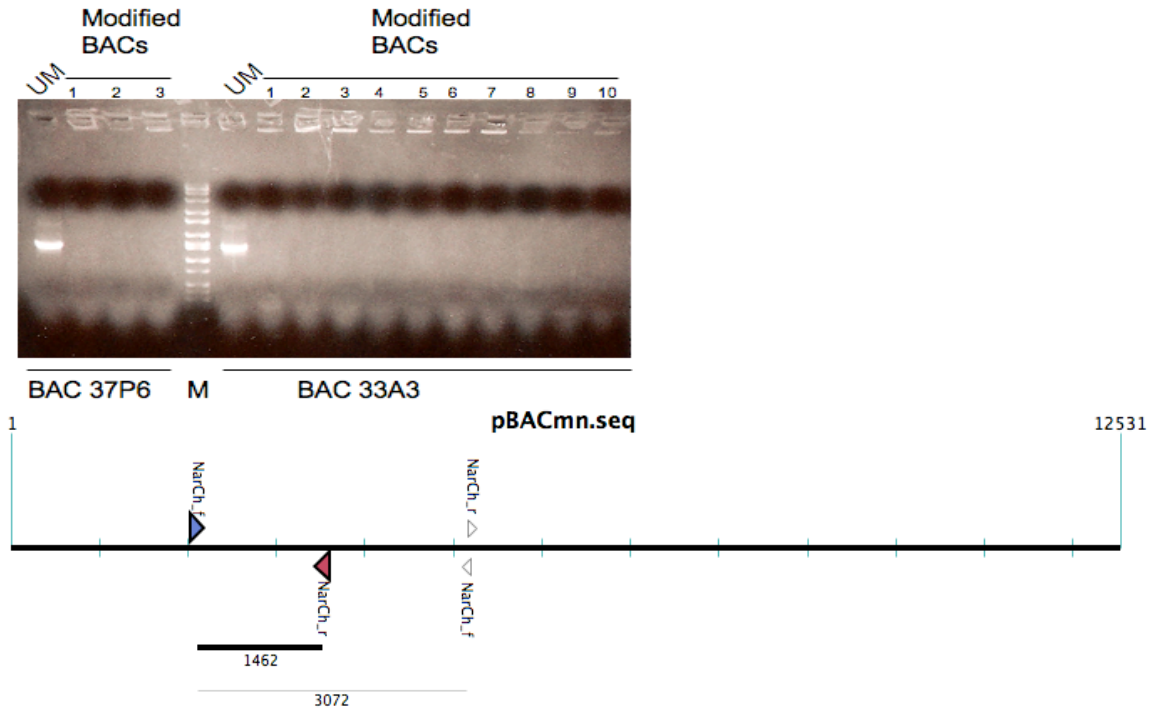
3.4.3.2 PCR to Show Replacement of the BAC Sequence with the Insert

After showing the presence of the insert in the modified BACs (Figure 24), we wanted to show the correct replacement of BAC sequence with our insertion. To do this we ran a PCR reaction for the region that was being replaced by the insertion (Figure 25A). If the replacement happened in this region, this time PCR should work only for the unmodified BACs and not for the modified BACs. Primer NarCh-f-2091 was designed to bind to the pBeloBAC11 vector sequence, upstream of the *nar8* sequence. Primer NarCh-r-3552 was designed to bind to the region of *nrt2* being replaced. Therefore if the replacement happened, NarCh-r-3552 will not have any priming site and PCR will not work. The product of this PCR is 1462 bp, as shown in Figure 25A. The diagram below, Figure 25A, shows the priming site of the primers on the pBACmn. The *nrt2* gene is 5022 bp and was inserted in 2389th position in pBACmn (see Figure 23).

In Figure 25B, PCR was done with a primer pair; both primers bind in the replaced region of *nrt2*. These primers amplify a 786 bp region of the *nrt2* gene. This PCR also should not give any product for modified BACs. However, only the BACs marked with the arrow did not produce any product. Therefore we only used those BACs for complementation. The reason for this could be that there may be unmodified BAC sequences retained in the bacterial cell along with the modified BAC. All of these bacterial clones were kept on LB+chloramphenicol+ kanamycin after the experiment. After many generations, the bacteria might have lost the unmodified version of the BAC. The primers used for this PCR were f-NarReplace and r-NarReplace.

We also did PCR with the same f-NarReplace primer paired with a primer that binds outside of the replaced region, but this time on the 3' of *nrt2* gene. This reaction also should not give any product for correctly modified BACs. We got the same results (Figure 25B, second gel). This result confirmed the result of first gel in Figure 25B. The primer that binds to 3' region of *nrt2* is pBACmn-6020.

A



B

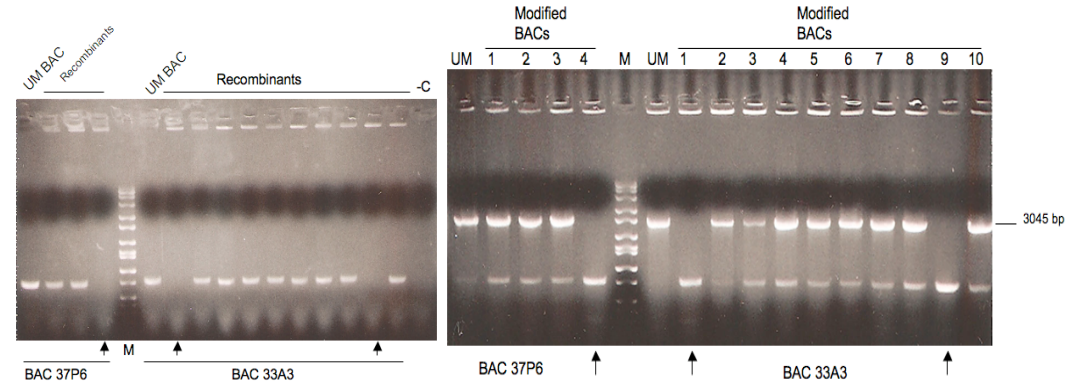
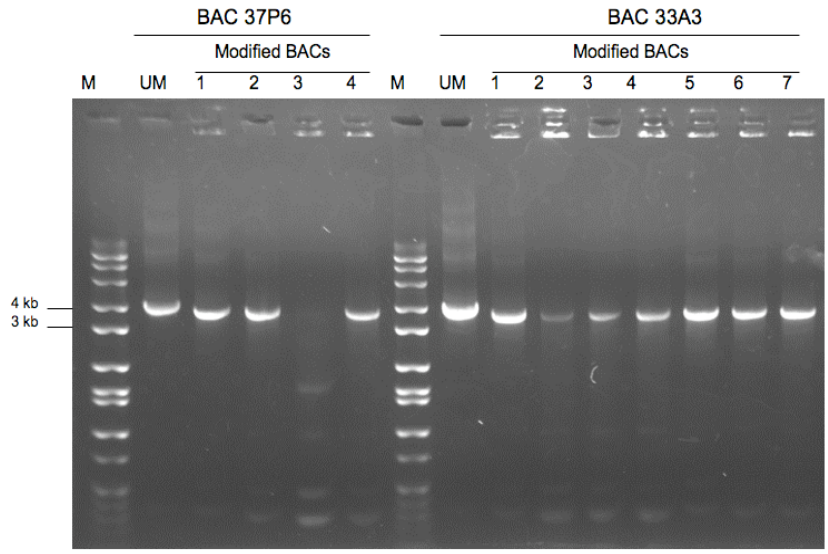


Figure 25. Correct replacement of the *nrt2* Sequence in the BACs. A. Since the primer NarCh-r-3552 will not have any binding site in the recombined BACs, no product is made. Only the unmodified BACs have product. **B.** The first gel shows the results with primers that bind in the vector and the replaced region of *nrt2*. The second gel shows the PCR results with primers that bind only in the replaced region. In both PCRs, the correctly modified BACs should not give any product, but some BACs did give a product. Therefore we continued only with those BACs marked with an arrow.

3.4.3.3 PCR with Primers Surrounding the Insert

We then designed primers that surround the insert in the BAC. We replaced a 3175 bp sequence of *nrt2* with a 3103 bp insert. Therefore the PCR should give a 72 bp smaller product for the modified BACs. These primers are NarCh-f-2091 and pBACmn-6020.



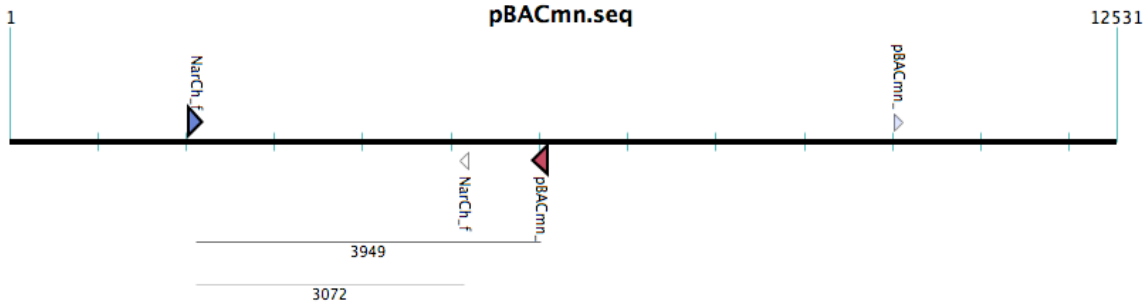


Figure 26. PCR with surrounding primers. A. All of the modified BACs should have a 75 bp smaller product then unmodified BAC. **B.** Priming site of the primers and the product. The product should be 3949 bp in the unmodified BACs.

3.4.4 Verification of Successful Recombination by Restriction Digestion

After checking the deletion in the modified BACs by PCR, we performed a restriction analysis, which is a better way of showing the recombination. The BACs marked with an arrow in the second gel in Figure 25B (BAC number 4 for BAC 37P6 and BAC number 9 for BAC 33A3), were used for restriction analysis and for the complementation experiments.

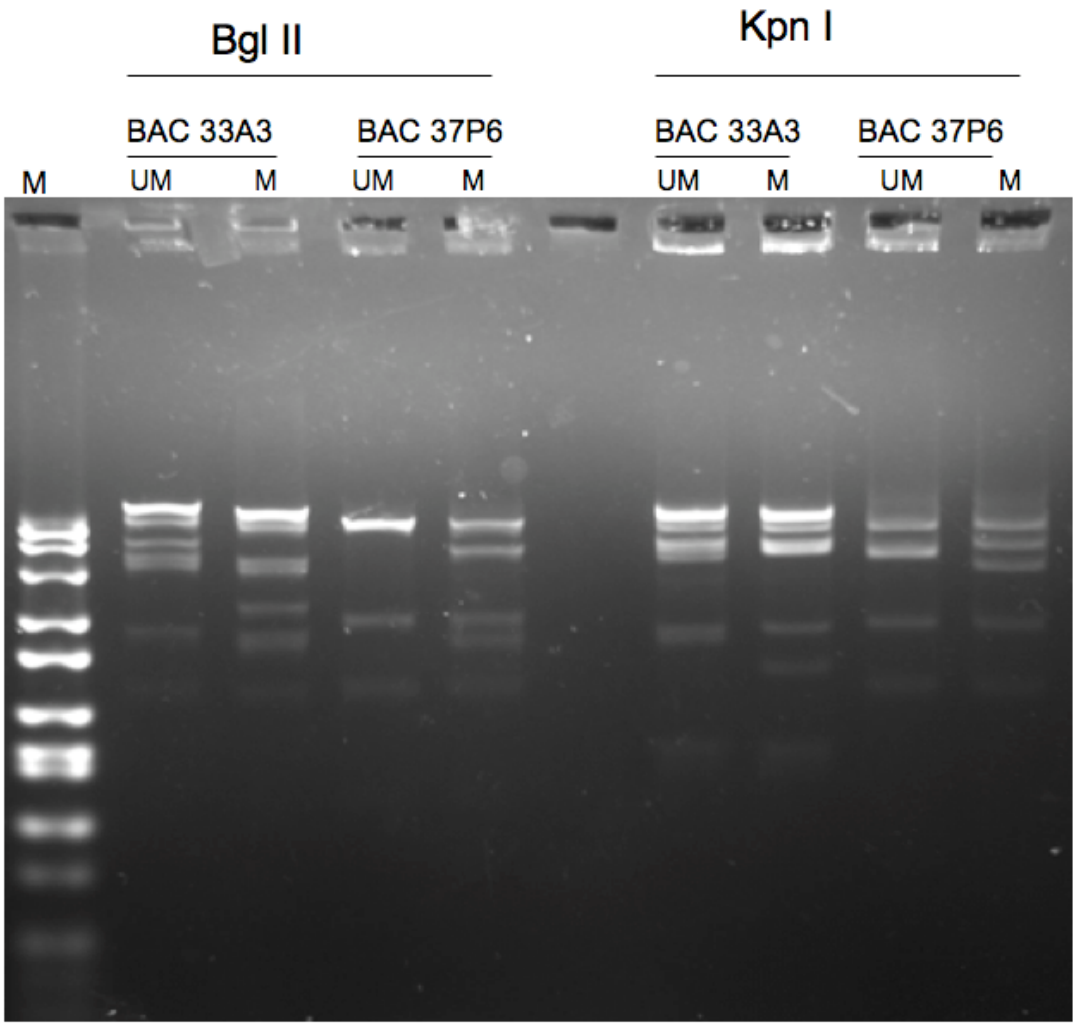


Figure 27. Verification of correct recombination by restriction analysis. M: modified, UM: unmodified. BACs were digested with 2 different enzymes to compare restriction pattern. In both digestions, both BACs show different restriction patterns for the modified and unmodified BACs, as expected.

3.5 Complementation of J1

In order to determine if the gene we identified with TAIL-PCR is required for gamete fusion, we have to transform the mutant with the wild type gene and see if it complements the mutation. The mutant J1 was therefore transformed with BAC 37P6 (modified and unmodified) to rescue the phenotype. The results and the methods used in each of these transformations are shown in table 4.

Table 4. Results of Complementation of J1

	Plate #	**BAC used**	**# of colonies on hygromycin**	**Mating Screening**	**Zygote Selection**
Transformation 1	1-5	37P6 UM	-	-	5,9,2,6,1 zygotes germinated
	6	37P6 UM+ 3 µg pHyg3	65	None mated	-
Transformation 2	1&2	5 µg 37P6 UM	-	-	None germinated
	3	5 µg Modified 37P6	25	None mated	-
	4	6 µg Modified 37P6	20	Clone-30 agglutinates with itself	-
	5	10 µg Modified 37P6	36	None mated	-
	6	UM 37P6+3 µg pHyg3	248	-	None germinated
Transformation 3	1-6	7 µg Modified 37P6	5,4,5,16,8,12, colonies respectively	None mated	-
	7	10 µg Modified 37P6	12	None mated	-
	8	5 µg 37P6 UM+ 1 µg pHyg3	38	None mated	-

In transformation 1, plates 1-5 were derived from a transformation with the unmodified BAC. Each zygote plate had a few green colonies which appeared to be germinated zygotes. In order to prove that these colonies were derived from germinated zygotes, we spread the whole colony onto TAP plates to obtain individual colonies. 2 potentially germinated zygotes from each plate 1, 2 and 5 were tested. From each zygote, 16 clones were picked (total of 96 clones). When they grew up, each progeny was mated both with mt^+ and *mt*-. Only if the germinating cells were progeny from zygotes, should we see colonies of both mating types.

The results of this random genetic analysis are shown in table 5. All of these tested germinated colonies, except for one from plate 3 had mixed progeny. We then wanted to see if any of the mt^- progeny could fuse (form zygotes) when mated with mt^+. Finally, we needed to determine if any of the fusion-competent mt^- progeny were Zeocin resistant. Any mt^- progeny that could grow on Zeocin should still have the *ble* insert (and should therefore still be mutant) unless it has been complemented by the BAC. All of the mating competent mt^- progeny of zygote 2 from plate 1 were found to be Zeocin resistant. Therefore we can say that this is a true zygote derived from the mating of a complemented J1.

In plates 2 and 3, none of the *mt*- progeny fused with mt^+, but some showed Zeocin resistance, suggesting they had the J1 mutation but not the BAC. These results now allow us to suggest that the insertion and fusion defective phenotype is linked (see the discussion).

Table 5. Analysis of random progeny of J1 zygotes

		# of mt^+ progeny	# of mt^- progeny	# of mt^- that are fused w/ mt^+	# of mt^- that are Zeo^R	# of mt^+ that are Zeo^R
Plate 1	**Zygote 1**	13	3	3	None	13
	Zygote 2	2	14	14	14	None
Plate 2	**Zygote 1**	4	12	None	11	4
	Zygote 2	4	12	None	4	None
Plate 3	**Zygote 1**	11	5	None	5	None
	Zygote 2	16	None	-	-	None

Plate 7 from transformation 1 was a co-transformation. All 65 hygromycin resistant colonies were mated with mt^+. None of these clones formed zygote pellicle.

In transformation 2, plates 1 and 2 were transformed with unmodified BACs. We did not obtain any zygotes germinating on these plates. 81 hygromycin resistant transformants were generated with the modified BACs (plates 3-5). None of these clones fused with mt^+. However, one clone, J1-30, showed a new phenotype which will be discussed below.

Plate 6 of this experiment was a co-transformation. We had 248 hygromycin resistant colonies. These transformants were collected and mated with mt^+. The mating mixture was plated on 4% plates for zygote selection, but we did not get any zygote germination

In transformation 3, we had fewer transformants then we had in transformation 2. In transformation 3 we did 8 transformations versus 6 in transformation 2. Because we used

more cells to do the transformation, the autolysin amount might not have been sufficient to remove the walls. None of these transformants fused with *mt*$^{+}$.

The clone, J1-30 mentioned above was found to agglutinate with itself and appeared to form a zygote pellicle. We wanted to find out if these pellicles were formed from true zygotes. We plated J1-30 cells that were mating with themselves on 4% TAP plates to see if any zygotes germinate. We also mated these J1-30 cells with *wt mt*$^{+}$ gametes. In neither of these cases did we see any zygote germination. (As a control, we also plated equivalent numbers of mated uncomplemented J1 X *wt mt*$^{+}$ gametes and again saw no germination). Therefore, we hypothesized that J1-30 formed a zygote wall after isoagglutination even though there is no fusion; but we do not know what caused this interesting phenotype. It could be that when we transformed J1, the overexpression of the gene caused a novel phenotype that is not found in literature or the BAC might have inserted into the genome at another site, creating a second mutation.

3.6 Determination of Mating Structure Adhesion in J1 by SEM

To further characterize this mutant and to determine if the phenotype we saw by phase contrast microscopy could also be observed by scanning electron microscopy (SEM), we prepared these cells and observed them for mating structure adhesion. Figure 28 shows both phase contrast and scanning EM views of this mating structure adhesion in a mating of J1 with *wt mt*$^{+}$. We can see that the mating structures are adhering but there is no fusion. Therefore we can say that J1 has a defect in the latest stage of fusion, as seen in *gam* mutants.

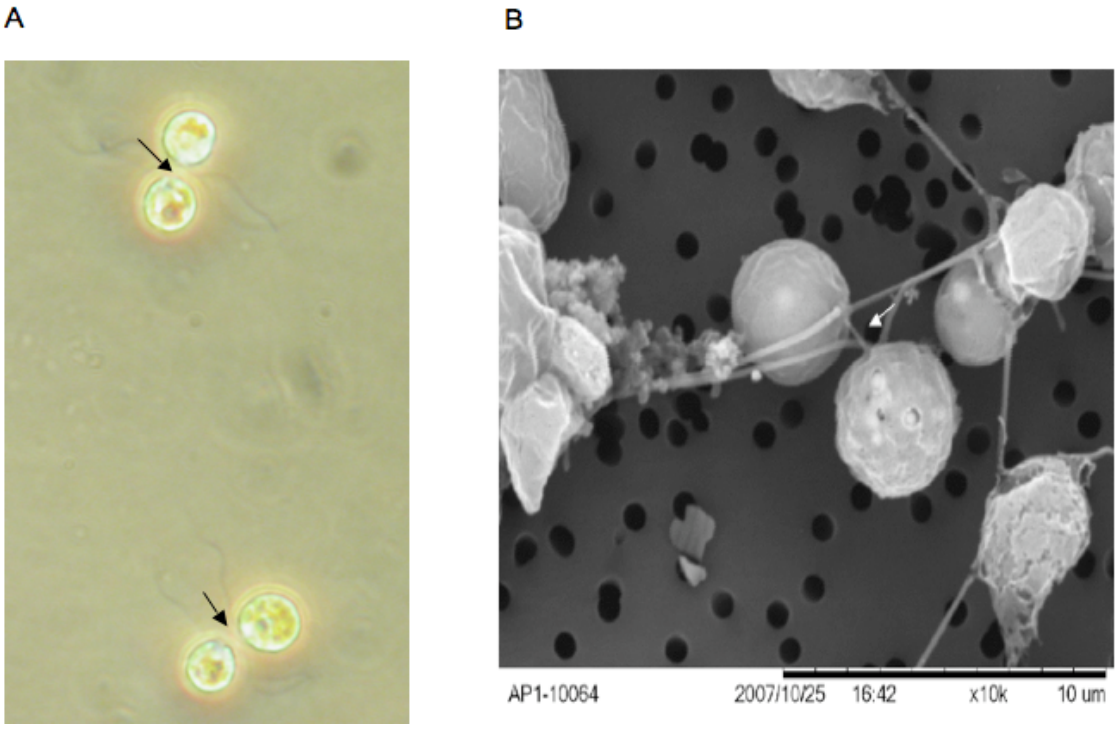


Figure 28. Mating structure adhesion in J1. A. Phase contrast image of adhering gamete pairs. Black arrows indicate the region where the mating structures adhere. **B.** SEM image showing the mating structure adhesion in a pair of gametes. The white arrow points to the mating bridge formed between the two gametes.

3.7 Is *FusM* Intact in Our Mutants?

Generative cell specific 1 (GCS1) was identified in lily and also found to have a homologue in *Chlamydomonas* (Mori et al., 2006). GCS1 is required for fertilization in angiosperms. Mutant male gametes are sterile. We wanted to determine if *FusM* (the homologue of GCS1 in *Chlamydomonas*) might have been deleted in any of our mutants. We did a PCR to amplify a region close to the 3'-end of the gene. As figure 29 shows, this region was intact in all the mutants. Primers GCS1-F and GCS1-R were used.

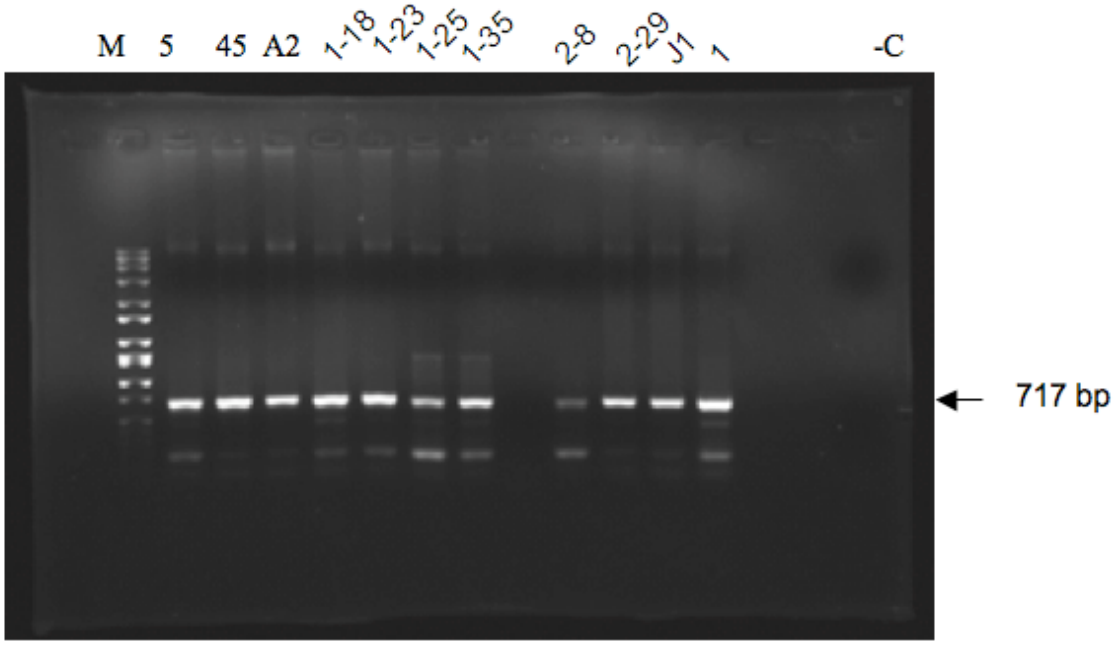


Figure 29. Amplification of the *fusM* sequence.

3.8 J1-30 and its Phenotypic Analysis

J1-30 was isolated from a transformation of J1 with the modified BAC 37P6 in transformation 1. When kept in N-free medium, J1-30 cells agglutinate with themselves and form pellicles (Figure 30 B and C). If we fix these isoagglutinating J1-30 cells, we find that they do not form pairs. However, when they are mated with mt^+ cells and fixed, they show the phenotype of the original J1 mutant; they can form pairs (Figure 30A). At this point we can only say that when the J1-30 cells have isoagglutinated for several hours, they stick to each other by an unknown mechanism and form what looks like pellicle. We suggest that cells may be forming true pellicle because their interaction has somehow lead to signals causing them to activate the zygote developmental pathway and causing them to form zygote walls.

The only known mechanism that would lead to the cells forming pellicle is the formation of zygotes by normal mating or by transformation of each mating type cells with the opposite mating type homeodomain transcription factors that are involved in zygote development. Gsp1 is a homeodomain protein expressed in mt^+ cells and Gsm1 is expressed in mt^- cells. After fusion, these two transcription factors form a heterodimer and initiate zygote wall formation. Mt^- cells that are transformed with the mt^+-specific homeoprotein Gsp1 form pellicles without fusion (Zhao et al., 2001a). Also mt^+ cells that are transformed with mt^- specific homeoprotein Gsm1 also form zygote walls (Goodenough et al., 2007). J1-30 has the same phenotype, but at this time we do not know the mechanism that initiates the zygote wall formation in these cells.

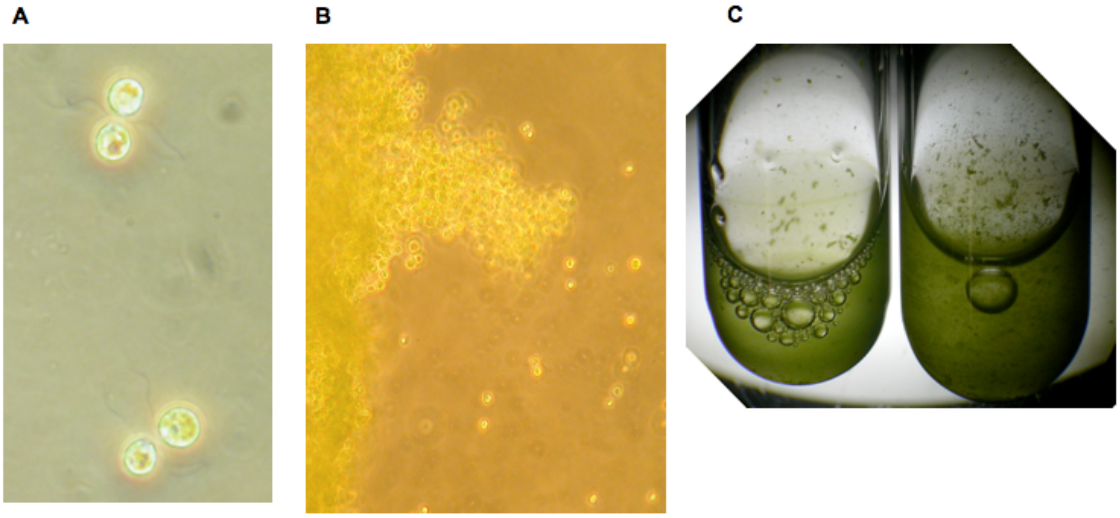


Figure 30. Phenotypic analysis of mutant J1-30. A. J1-30 cells form pairs with mating type plus cells. **B.** Phase contrast image showing pellicles. **C.** Tube on the left shows J1-30 cells which formed pellicles with themselves in N-free medium. Tube on the left is zygote pellicles after wt mating.

3.9 Genetic Mapping Results of *gam-10*

A different approach was also taken to determine the location of genes involved in gamete fusion. The previously isolated temperature sensitive mutant, *gam-10* (Forest, 1983) was crossed to mapping strains obtained from the *Chlamydomonas* Culture Collection. To perform the mapping, *gam-10* cells were mated with each of these strains. (see Table 3). Results of tetrads analysis are shown in table 6. Each zygospore colony progeny was transferred to scoring (selection) plates. Spotting a small amount is important because if too many cells are spotted, scoring might not be conclusive. We then determined if the progeny were fusion-defective. Cells from each of the progeny were mated with wild type mt^+ and mt^- at room temperature to determine their mating type. After mating type was determined, mating type minus progeny were mated at the restrictive temperature (32°C) to determine if they were temperature sensitive. To determine the linkage of the genetic markers and fusion defective phenotype (*gam-10*), each marker and the fusion defective phenotype were scored. If the genetic marker being scored and the *gam-10* were on the same chromosome, we expect to see high numbers of parental ditypes (PD) in the tetrads being tested. If the genetic marker and the *gam-10* were not on the same chromosome, we expect to see high number of non-parental ditypes (NPD). If high number of tetratypes (T) and few NPDs are seen, it means the genetic marker and the *gam-10* are not linked.

Table 7 shows the result of one of these analyses as an example.

Table 6. Result of tetrad analysis

Strain	Genetic Marker	Phenotype	Linkage Group	Result	# of Tetrads
CC27	*ac31*	Chlorophyll deficient, pale green; forms small colonies on minimal medium.	V	2 PD, 3 NPD, 3 T	11
	nic1	Requires nicotinamide	XV	1 PD, 5 NPD, 1 T	
	pf12	Paralyzed flagella (not easy to score)	II	Pf marker was not scored	
	thi10	Requires thiamine	VI	3 PD, 3 NPD	
CC 641	*act2*	Resistant to cycloheximide, but often doesn't show linkage to markers on the left arm	VI	3 T	15
	nic1	Requires nicotinamide	XV	1 PD, 2 T	
	pf12	Paralyzed flagella	II	2 NPD, 1 T	
CC 1768	*gln1*	Cannot use glutamine as sole N-source	XII/XIII	3 PD, 1 NPD, 1 T	17
	pf27	Paralyzed flagella	XII/XIII	3 PD, 2 NPD, 1 T	

Table 7. Tetrad analysis of *gam-10* X CC 1768

Tetrad #	*Flagella N: normal P: paralized*	*Growth with Glutamine N: No Y: Yes*	*Mating Type*	*Mating at 32 ℃*	*Mating Phenotype*	*Conclusion for fla*	*Conclusion for glu*
1_1	N	N	+		gam	PD	PD
1_2	P	Y	-	Y	wt	PD	PD
1_3	P	Y	-	Y	wt	PD	PD
1_4	N	N	+		gam	PD	PD
2_1	N	N	+		gam	PD	PD
2_2	N	Y	-	Y	wt	NPD	PD
2_3	P	Y	-	Y	wt	PD	PD
2_4	P	N	+		gam	NPD	PD
3_1	N	N	+				
3_2	P	Y	+				
3_3	P	Y	-	Y	wt	PD	PD
3_4	N	N	-	N	gam	PD	PD
4_1	P	N	?				
4_2	P	Y	+				
4_3	N	N	+	Y	wt	NPD	NPD
4_4	N	Y	-	N	gam	PD	NPD
5_1	N	N					
5_2	N	N					
5_3	N	N					
5_4	P	Y					
6_1	N	N	+				
6_2	P	Y	-	Y	wt	PD	PD
6_3	P	Y	+				
6_4	N	N	-	N	gam	PD	PD

Table 7. continued

					gam	PD	PD
7_1	N	N	-	N			
7_2	P	Y	+				
7_3	N	N	-	N	gam	PD	PD
7_4	P	Y	+				
8_1	P	N	-	Y	wt		NPD
8_2	P	Y	-	Y	wt		PD
8_3	N	Y	+	N	gam	PD	NPD
8_4	N	N	+	N	gam	PD	PD
9_1	N	N	-	N	gam	PD	PD
9_2	P	Y	+				
9_3	N	N	+				
9_4	P	Y	-	Y	wt	PD	PD
10_1	P	N	-	Y	wt	PD	PD
10_2	N	Y	-	N	gam	PD	NPD
10_3	N	Y	+				
10_4	P	N	+				
11_1	P	Y	-	N	gam	NPD	NPD
11_2	P	N	-	Y	wt	PD	PD
11_3	N	N	+				
11_4	N	Y	+				
12_1	P	Y	-	N	gam	NPD	NPD
12_2	P	Y	-	N	gam	NPD	NPD
12_3	N	N	+		wt	NPD	NPD
12_4	N	N	+		wt	NPD	NPD
13_1	N	N	-	N	gam	PD	PD
13_2	P	Y	+				
13_3	P	Y	+				
13_4	N	N	-	Y	wt	NPD	PD

Table 7. continued

14_1	N	N	+				
14_2	N	N	-	N	gam	PD	PD
14_3	P	Y	-	Y	wt	PD	PD
14_4	P	Y	+				
15_1	N	N	-	N	gam	PD	PD
15_2	N	N	+				
15_3	P	Y	-	Y	wt	PD	PD
15_4	P	Y	+				
16_1	P	Y	-	N	gam	NPD	NPD
16_2	N	N	+			NPD	NPD
16_3	P	N	-	N	gam	NPD	PD
16_4	N	Y	+			NPD	PD
17_1	P	Y	-	Y	wt	PD	PD
17_2	N	N	-	N	gam	PD	PD
17_3	P	N	+				
17_4	N	Y	+				

The *gam-10* gene is not linked to the mating type locus. However the fact that this gene is sex-limited (expressed only in mt^-) complicates the traditional Mendelian analysis. (In *Chlamydomonas* we can do complete tetrad analysis. This means, that all 4 meiotic products are obtained and can be individually analyzed.) However, when mapping this type of autosomal sex-limited gene, you can only see the mutant phenotype when the gene has segregated with the mating type minus locus (when it is present in a mt^- cell). If the *gam-10* gene segregates with the mating type plus locus (when present in a mt^+ cell) it is not expressed. Therefore, we often do not know if a mt^+ cell is carrying the mutant allele. The only time we can be sure mt^+ is carrying the allele is if both of the mt^- progeny are wild type (indicating that both copies of the mutant allele are in the mt^+ progeny) or if we cross the mt^+ cell to a wild type and recover the mutant phenotype in

the F2 progeny. Because of this complication, when we analyze a tetrad, we frequently do not know which 2 zygospores carry the mutant gene. In these cases, we cannot determine if a tetrad is a tetratype or a non-parental ditype. However, if a tetrad is a parental ditype (all 4 products are like the parents, the mt^+ parent and progeny are wild type and the mt^- parent and progeny are mutant) we can determine this from our F1 analysis.

An example of this situation is seen in tetrad 17. In any tetrad you should see a 2:2 segregation of the *gam-10* gene; however we only see one zygospore expressing the *gam-10* phenotype. We therefore know that one of the *gam-10* genes is found in zygospore #2, which shows the mutant phenotype. All of the other 3 zygospores can mate including zygospore #1, which is mt^- and can mate at 32 °C. This means zygospore #1 contains the wild type version of the *gam-10* gene. Therefore the mutant allele has segregated with one of the mt^+ zygospores, but we do not know which one. With just the information from the F1 progeny, we cannot determine if tetrad 17 in this cross is a tetratype or non-parental ditype. To determine which of the mt^+ zygospores is carrying the *gam-10* gene, we have to cross zygospores #3 and #4 with *wt* mt^- and analyze the F2 progeny.

According to the results presented above, only tetrads 1, 2, 7, 8, 12, and 16 are conclusive. For the *pf27* marker there are 3 PDs, 2 NPDs and 1 T. In a different cross, 3 PDs, 1 NPD, 2 Ts for *pf27* marker, and 3 PDs, 1 NPD, 2 Ts for *gln1* marker were observed.

In summary, from these mapping results we cannot map the *gam-10* gene to any linkage group (chromosome) we have tested. From the results of *gam-10* X CC27 cross, we can say that *gam-10* is not linked to linkage groups V, VI or XV. Crosses done with CC 641

and CC 1768 also have high numbers of non-parental ditypes; therefore *gam-10* is not linked to any of the markers tested. But to be more conclusive we have to analyze more tetrads for CC 641 and CC 1768.

CHAPTER 4

DISCUSSION

Mating in *Chlamydomonas* has been extensively studied (see figure 31 for a description of the stages in this process). However, as in fertilization in other organisms, we know very little about the genes/proteins that are necessary for the final stage, fusion of the gametes.

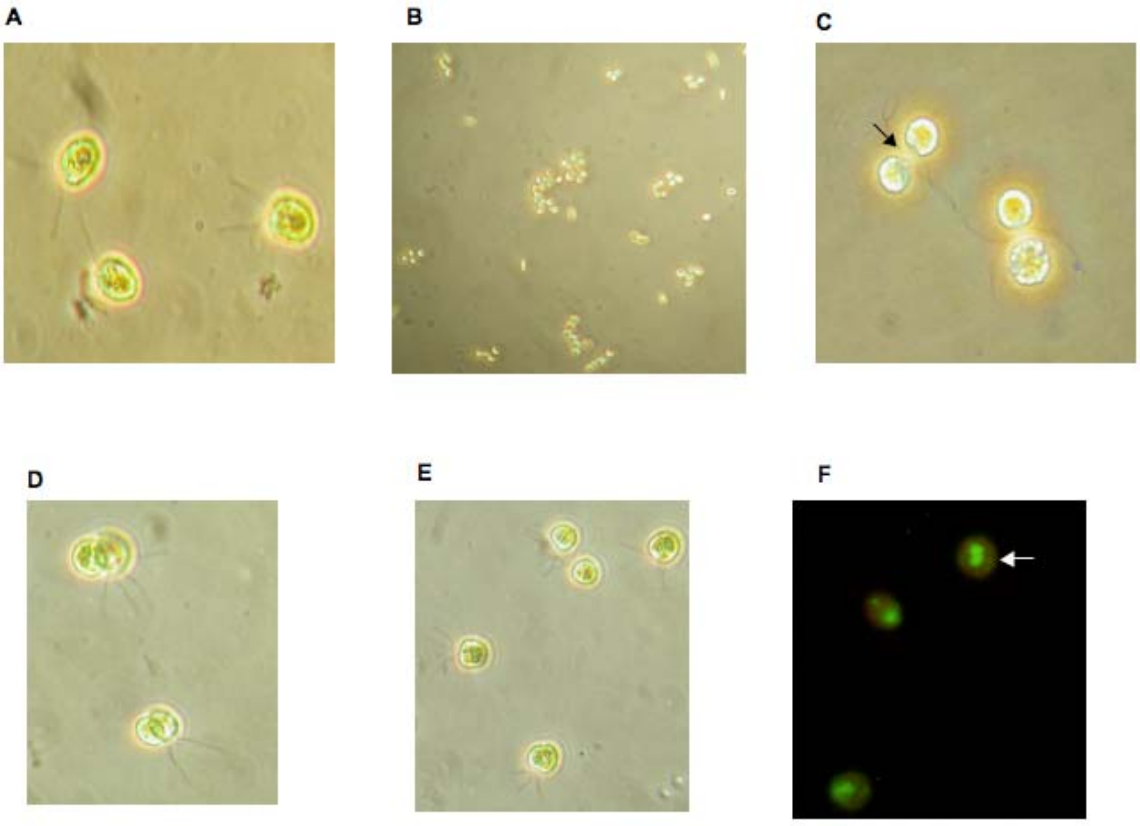


Figure 31. Mating in *Chlamydomonas.* A. Biflagellated gametes. **B.** Agglutinating gametes. **C.** Pair formation. Arrow is pointing to the region where mating structures adhere. **D.** Two zygotes almost completed fusion. **E.** Quadriflagellated zygotes completed fusion. **F.** White arrow is pointing to a zygote which has 2 nuclei. Nuclei will fuse in an hour after fusion.

We therefore applied forward genetics to identify genes that are involved in gamete fusion in *mt*$^-$ *Chlamydomonas* cells. Cells of the streptomycin resistant strain, *sru*-2-23 *mt*$^-$ were randomly mutagenized by DNA insertion of pSP124S. Overall, 7 fusion defective mutants were generated in 3 independent transformations. In earlier work, Ms. Tammy La and Ms. Jane Lam each created one mutant in independent transformation experiments. We showed that all of the insertional mutants are able to recognize *mt*$^+$ cells, transmit signals, remove cell walls and adhere to *mt*$^+$ partners, suggesting that our mutants are blocked at the last stage of mating. We also isolated mutants that have defects in mating partner recognition, but those mutants were not the subject of this work. We believe they may have mutations in gamete differentiation or in agglutinin synthesis. Further, a mating type switch mutant (clone-1) was isolated in which we think the insertion might have disrupted the *mid* gene whose function is to activate *mt*$^-$ gene expression and to repress *mt*$^+$ gene expression.

We showed the insertion number for each of the fusion defective mutants by Southern blot analysis. According to our Southern blot results, Clone-5 has 2 or 3 insertions. All other mutants except 1-35 and 2-8 (which showed no bands on the Southern blots) appeared to have only one insertion.

Since traditional genetics could not be performed with these fusion defective mutants to show the linkage of the insertion and mutations, we attempted fusing the mutants by artificial cell fusion using polyethylene glycol, but we were not successful in causing these cells to fuse. But we will be able to show the linkage using the progeny of complemented J1 (discussed below).

Following determination of the number of insertions, we tried to sequence the flanking genomic region of the insertions. We found TAIL-PCR and RESDA-PCR to be the most efficient techniques for cloning of flanking regions. We were able to clone the flanking region of the clone-5 and J1 insertion sites. Since clone-5 has more then one insertion, we cannot conclusively determine that we sequenced the flanking region of the insertion that caused the fusion defective phenotype. The mutation causing the phenotype might be in a different region in the genome. It might be possible to do this genetics if we can complement the mutation. In contrast, mutant J1 had only one insertion. Our results suggest that the mutation is linked to the insertion.

The flanking genomic region we cloned for mutant clone-5 is in scaffold-44. The insertion occurred in position 523923. We wanted to determine if any deletion occurred at the region of the insertion; PCR analysis did not show any deletion (the results are not shown here). Version 3 of the *Chlamydomonas* genome does not have any gene models predicted at this position. Version 2 of the genome had a putative reverse transcriptase where the insertion happened, but this gene model (C_2470009) was removed from version 3. Abe et. al showed that this gene was upregulated in gametogenesis (Abe et al., 2004). The gene model (C_440054) upstream of the insertion is a predicted protein with a carboxypeptidase domain. Downstream of the insertion is a putative GTPase, elongation factor-like protein. This GTPase was annotated as a gwH.44.48.1 in version 3 of the *Chlamydomonas* genome. It is predicted to be a membrane GTPase that might be involved in stress response in *Novosphingobium aromaticivorans* (a gram negative aerobic bacteria). If it is involved in stress response, it might be involved in signal transduction during gametogenesis in *Chlamydomonas*. We obtained BAC 33A3 that

covers all 3 of the gene models. We modified BAC 33A3 by recombineering and it can now be used for selection of transformants after transformation of clone-5 cells.

In contrast to mutant clone-5, the mutant J1 had only one insertion which disrupted a predicted gene (C_370109) in scaffold 37. This gene has two gametic ESTs and has no known functional homologues in any other organisms. However, this gene it is annotated as generating an expressed protein in the higher plants *Arabidopsis*, rice and the green alga *Volvox*. We used prediction tools to analyze the protein sequence of C_370109. Toppred prediction shows that the putative protein might have a single transmembrane domain.

The gene model downstream (C_37000110) is annotated as having function in oxygen transport. We performed PCR for this gene to see if there was any deletion occurred; we did not see any deletion. Therefore we do not think this gene model is involved in fusion.

Upstream of the gene model containing the insertion, there is a gene model which has kinase activity but only has vegetative ESTs (C_370024). This kinase has a homologue in male germ cells of mouse, but the exact function of this kinase is not known. This protein is found in the flagellar proteome of *Chlamydomonas*; therefore we could suggest that it might be involved in signal transduction during mating.

To determine if gene C_370109, which was disrupted by the insertion, was responsible for the phenotype of mutant J1, J1 was transformed with BAC 37P6, which contains the genes C_370109 and C370024, to see if either of these genes could rescue the phenotype. Following transformation of mutant J1 with BAC37P6, mating of the transformants resulted in production of zygotes, suggesting that mutant J1 was complemented. We performed a random progeny analysis of these zygotes and showed that each zygote

produced both mating type plus and minus progeny confirming that they were indeed zygotes. In the progeny, we had *mt*$^-$ Zeocin resistant and mating competent clones. Random progeny analysis also showed Zeocin resistant *mt*$^+$ cells, again confirming that the cells obtained were the products of meiosis. These results confirmed that we rescued the phenotype of J1. We now can use the progeny of complemented J1 and do the genetic analysis that we couldn't do with the original mutants, to show linkage of the insertion and the fusion defective phenotype. In the progeny of these tetrads we had *mt*$^-$ Zeocin resistant fusion defective zygospores (the original phenotype of the mutants) and *mt*$^+$ Zeocin resistant cells. There are 2 ways we can show linkage from these progeny. If all of the fusion defective *mt*$^-$ zygospores are also Zeocin resistant, this indicates the mutation and the insert are linked. All of the *mt*$^-$ zygospores derived from our random genetic analysis are indeed Zeocin resistant. Secondly, we will cross the *mt*$^+$ Zeocin resistant cells with *wt mt*$^-$. All of these crosses should produce *mt*$^-$ Zeocin resistant fusion defective progeny.

The final question remaining is which of the two genes in BAC 37P6 rescued the phenotype. Most likely gene C_370109 rescued the phenotype, because the insertion is in this gene and because it has been shown to be expressed in gametes (it has gametic ESTs while the other gene appears to only be expressed in vegetative cells). To conclusively demonstrate that the disruption of gene C_370109 is responsible for the fusion defective phenotype of the J1 mutant of *C. reinhardtii*, we will use just the wild type version of gene C_370109 for transformation of mutant J1.

For complementation of mutants of *Chlamydomonas* with BACs, a novel construct was made containing the Hygromycin resistance gene as a selection marker. Such a construct

has never been used before for *Chlamydomonas* BACs. We created a fusion cassette by fusing a bacterial and a *Chlamydomonas* antibiotic resistance gene. We used this fusion cassette to delete the *nar8* gene in the BAC. We can now use the same technique to delete either one of the gene models (C_370109 or C_370024) and use the modified BAC to transform J1 cells. The transformants can either be selected on hygromycin or without any antibiotic selection (we can use all of the cells to mate with mt^+ and select for zygotes).

In this study, we also isolated a strain with a novel phenotype which has never been reported in the literature. This strain (J1-30) was isolated from a transformation of mutant J1 with the modified BAC 37P6. Cells of this new strain, J1-30, agglutinate and form pellicles with itself. At this time we do not know the cause of this phenotype. Two possible explanations for this novel phenotype are that we introduced a new mutation into J1 or we are seeing a phenotype resulting from overexpression of one of the genes in the BAC. We have checked to see if the cells forming these pellicles would survive chloroform vapor, but they did not. This result indicated that the self-agglutinating cells did not form true thick zygote walls around them which should have protected cells from chloroform vapor. Further studies that are beyond the goal of this thesis will be needed to determine the genetic defect that caused this new phenotype.

In conclusion, I have isolated insertional fusion defective, mt^- mutants, showed the insertion number for each mutant by Southern blot, sequenced the flanking region in two of the mutants and complemented one of the mutants.

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