Proceedings of The National Conference
On Undergraduate Research (NCUR) 2017
University of Memphis, TN
Memphis, Tennessee
April 6-8, 2017

Molecular Genetic Analysis of 5'-3' Exoribonuclease (*Xrn*) Mutants in the Model Green Alga *Chlamydomonas reinhardtii*

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Abstract

Organisms regulate the expression of genes, which can help control development and responses to environmental changes. In the case of plants and algae, nucleus-encoded regulatory proteins help control photosynthesis in chloroplasts, and some of these regulatory proteins control the stability of chloroplast mRNAs that in turn alter protein abundance. Nuclear genes predicted to regulate chloroplast gene expression have been investigated in the single-celled alga *Chlamydomonas reinhardtii*, a model organism used to study photosynthesis. Preliminary data suggest that accumulation of some chloroplast mRNAs is controlled, in part, through degradation by a 5'-3' exoribonucleolytic activity. Sequence analysis predicts that *C. reinhardtii* has four *5'-3' exoribonuclease* (*Xrn*) genes. Two appear to encode Type 1 Xrn proteins, typically targeted to the cytoplasm, while the other two appear to encode Type 2 Xrn proteins, typically targeted to the nucleus. To investigate the function of these predicted exoribonucleases we have obtained ten *C. reinhardtii* mutant strains, each with a predicted DNA insertion into one of the *Xrn* genes. The mutants are from the recently reported Indexed, Mapped Mutant Library (CLiP) in *C. reinhardtii*. Our initial growth tests confirmed that all ten mutants contain the selectable paromomycin resistance gene *AphVIII*, and we are using an initial PCR screen to test location of the inserted DNA within the *Xrn* genes. Mutants that contain the predicted insertion within a targeted *Xrn* gene are being analyzed for changes in growth phenotypes compared to a wild-type control, when grown under standard conditions. The long-term goal is to assess molecular phenotypes to identify changes in abundance or processing of candidate RNAs so as to understand the function of *Xrn* genes. Ultimately, RNA-Seq (transcriptomic) analysis would be used to test mutants for genome-wide changes in RNA abundance and sequence. Complete analysis of these mutants will provide a better understanding of the function of *Xrn*'s in algae and possible impacts on photosynthesis.

Keywords: *Chlamydomonas*, 5'-3' exoribonuclease, RNA stability

1. Introduction

The regulation of gene expression is important in all organisms. It can control biological processes, including growth, developmental, and responses to environmental conditions and stress. In plants and algae the process of photosynthesis is highly regulated to control the amount of absorbed solar energy, stored high-energy chemicals, and carbohydrate synthesis, while minimizing damage from excess input energy. To help control photosynthesis, plants and algae rely, in part, on nucleus-encoded regulatory proteins that modulate photosynthesis in chloroplasts. Nucleus-encoded regulators are imported into chloroplasts where they can control different steps of chloroplast gene expression, including RNA processing and stability, translation, and protein stability[1]. Using genetic and molecular approaches, our group is studying nucleus-encoded regulators of chloroplast RNA stability in the single-celled eukaryotic green alga *Chlamydomonas reinhardtii*, a model organism for photosynthesis.

The *C. reinhardtii MCD1* is an example of a nuclear gene that specifically regulates the stability of the *petD* chloroplast mRNA, which encodes the essential SUIV protein of the cytochrome b_6/f complex that is needed for the

photosynthetic electron transport in all plants and algae[2, 3]. The mRNA degradation mechanism for *petD* appears to occur processively by a 5'-3' exoribonucleolytic activity[4]. Since there are no ribonucleases (RNases) encoded in the *C. reinhardtii* chloroplast genome, all chloroplast RNases must be encoded elsewhere, translated in the cytosol, and imported into chloroplasts[5]. 5'-3' exoribonucleases (Xrn's) are known to degrade mRNAs processively in a 5'-3' direction, are common to all eukaryotes, and are targeted to multiple cellular locations[6]. Xrn proteins degrade RNAs by breaking the phosphodiester bond on the 3' side of the ribose sugar and before the 5' phosphate on the downstream nucleotide. This cleaves off a single nucleotide from the 5' end of an unprotected RNA[7]. Xrn's have important functions in regulating gene expression by controlling mRNA abundance. In turn, this can alter the amount of the encoded protein. The combination of genomic sequence analysis and genomic library screening have identified four predicted *Xrn* genes in the nuclear genome of *C. reinhardtii*[8, 9]. Previous work suggests that the *CrXrn1* gene (Cre06.g280050) may utilize an alternative splicing process that we hypothesize to generate a chloroplast-targeted Xrn. This may provide the 5'-3' exoribonucleolytic activity that degrades the *petD* mRNA in the chloroplast[10].

Our group is investigating the four predicted nuclear *Xrn* genes for their function and to determine if one or more might regulate chloroplast gene expression in *C. reinhardtii*. As is common in eukaryotes, each of the four predicted *C. reinhardtii* Xrn proteins can be categorized into either Type 1 or Type 2 Xrn's (see Table 1). Both types have 5'-3' exoribonuclease activity, and their functions and RNA targets are based, in part, on their cellular location. Type 1 Xrn proteins are typically targeted to the cytoplasm while Type 2 Xrn proteins are typically targeted back to the nucleus[11]. To better understand the function of each of the four *C. reinhardtii Xrn's* genetic mutants need to be identified and characterized phenotypically. A recent Indexed, Mapped Mutant (CLiP) Library for *C. reinhardtii* has become available, and it has ten predicted *Xrn* mutants[12, 13]. This mutant library contains many different mutant strains (~37,000) of *C. reinhardtii* with a ~3-kb inserted DNA cassette, predicted to be in many genes throughout the genome[13]. These typically have one insert per mutant. Understanding the phenotypes for these potential mutants will aid in deducing the function of Xrn's and roles in *C. reinhardtii* biology and possibly photosynthetic processes. The ten mutant strains that contain a predicted insertion into any of the four *Xrn* genes were obtained from the CLiP library. This report describes the initial genetic and phenotype characterization of these ten mutants.

2. Methods and Materials

2.1. Algal Strains And Growth Conditions

All strains of *Chlamydomonas reinhardtii* algae were maintained on Tris Acetate Phosphate (TAP) media in liquid cultures or on TAP agar plates[14]. Liquid cultures were grown under constant light (100 µE/m^2/sec) at room temperature (23ºC) while shaking at ~110 rpm and maintained by transferring 0.5 ml of an established culture into 50 ml of fresh TAP media weekly. Agar (1.5%) TAP plates were maintained under continuous light (100 µE/m^2/sec) at room temperature.

The ten candidate *Xrn* mutants (Table 1) and corresponding wild-type (WT) strain (CC-4533) were obtained directly from *C. reinhardtii* Indexed, Mapped Mutant (CLiP) Library website[15]. To confirm presence of the transformed insert DNA with the paromomycin antibiotic resistance gene (*AphVIII*) from *Streptomyces rimosus*, all strains were grown for one week on TAP agar plates with either 20 µg/ml or 30 µg/ml paromomycin (Fisher Scientific).

Table 1. List of predicted *C. reinhardtii Xrn* mutants, proposed gene name, *C. reinhardtii* genome locus identification number (from the *C. reinhardtii* genomic database at Phytozome[9]), Chromosome number, predicted Xrn protein Type (XRN1 or XRN2) based on predicted protein sequences, CLiP mutant strain number, and predicted location of insert DNA within an *Xrn* gene.

Proposed Gene Name	***C. reinhardtii* Locus Identification (Phytozome)[9]**	**Chromosome**	**Xrn Type**	**CLiP Mutant Number[15]**	**Predicted Insertion Site[15]**
CrXrn1	Cre06.g280050	6	XRN1	RY0402.**105856**	Intron 13
" "	" "	" "	" "	RY0402.**117676**	Intron 12
CrXrn2	Cre16.g679900	16	XRN2	RY0402.**174346**	Intron 9
CrXrn3	Cre08.g373916	8	XRN2	RY0402.**114070**	Intron 3
" "	" "	" "	" "	RY0402.**171169**	Intron 4
" "	" "	" "	" "	RY0402.**190466**	Intron 10
" "	" "	" "	" "	RY0402.**197122**	Intron 15
CrXrn4	Cre03.g152100	3	XRN1	RY0402.**042261**	Exon 9 / Intron 9 overlap
" "	" "	" "	" "	RY0402.**078140**	3' UTR, 5' of polyA site
" "	" "	" "	" "	SG0182.**007713**	Intron 12 / Exon 13

Cell density growth curves in TAP liquid media (50 ml) and diluted spot plate assays on TAP agar were used to assess growth phenotypes under standard growth conditions. For each culture 50 ml of fresh TAP liquid media were inoculated with 0.5 ml of cells at 2 x 10^6 cells/ml at time "zero". Three independently grown replicates of each strain were tested using growth curves, and all cultures were grown for seven days under standard growth conditions (described above). Cell density (cells / ml) for all strains were determined every 12 hours over the 7-day growth curve using hemocytometer quantification[14]. Mathematical averages from the three independent cultures for each strain were calculated along with the standard deviation.

To independently confirm cell density data, diluted spot plate assays were prepared every 24 hours during the 7-day growth curve, from two representative replicates of each strain. For the "undiluted" samples, 10 µl of each culture were spot plated directly onto TAP agar. Samples from each culture were also diluted to "1/10", "1/100", "1/1,000", and "1/10,000" the original culture in sterile TAP agar, and 10 µl of diluted samples were spot plated onto TAP agar. Diluted spot plates were then incubated for five days under standard growth conditions to visualize growth. Plates were photographed to document amount of growth.

2.2. Genomic DNA Isolation And Polymerase Chain Reaction (PCR)

All *C. reinhardtii* genomic DNA was isolated using a modified method based on a small-scale isolation[16]. 50 ml of liquid TAP cultures were grown to mid-log phase (~2 x 10^6 cells/ml) under standard growth conditions (described above). Ten ml of this culture were centrifuged at 4,000 rpm for 5 min to pellet cells. Cell pellets were either used immediately for DNA isolation or frozen at -80°C for future extraction. A Proteinase K Buffer was prepared (0.01M Tris, pH 8.0, 0.01M EDTA, 0.01M NaCl) and 0.5 ml added to the cell pellet. After resuspension, Proteinase K (Fisher Scientific) was added to a final concentration of 200 µg/µl along with 12.5 µl of 20% SDS. The solution was incubated at 50°C for 2 to 3 hours (periodically inverting to mix) until the sample turned brown. Next, 550 µl of Tris-buffered phenol (pH 8.0) were added to the cells, gently mixed, and centrifuged at top speed for 5 min. The upper aqueous layer was transferred to a new tube, and 5 µl of RNase A (10 mg/ml) were added then incubated at 37°C for 30 min to degrade any contaminating RNA. Samples underwent a second phenol extraction, same as previously described but with 500 µl Tris-buffered phenol (pH 8.0), followed by a chloroform (500 µl) extraction. The DNA was precipitated with 50 µl of 3M sodium acetate and 300 µl of 100% isopropanol, followed by a 10 min centrifugation to pellet DNA. The DNA pellet was washed with 200 µl of 80% ethanol and air dried for 5 min. To further clean the DNA, the pellet was resuspended in 100 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0) and subsequently precipitated using 100 µl of NaCl/PEG solution (2M NaCl; 20% PEG, MW 8,000) and placed on ice for 10 min. The precipitated DNA was centrifuged for 10 min, and the DNA pellet was washed twice with 200 µl of 80% ethanol, once with 100 µl 100% ethanol, and air dried 10 min. Finally, the DNA pellet was resuspended in 30 µl TE (pH 8.0) and stored at -20ºC.

An initial genetic screen using polymerase chain reaction (PCR) was used to test mutants for the inserted DNA in the predicted location and within the correct *Xrn* gene. For this, 1 µl of isolated *C. reinhardtii* genomic DNA (described above) was added as template to a 25 µl PCR. In addition, each 25 µl PCR contained 1 X GoTaq DNA Polymerase Thermal "Flexi" Buffer (Promega Corporation), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 120 ng Forward Primer, 120 ng Reverse Primer, 1.2% DMSO, and 0.2 µl GoTaq DNA Polymerase (5 units/µl; Promega Corporation). PCR was performed on a Bio-Rad C1000 Thermal Cycler (Bio-Rad Corporation) using the following thermal cycling program: 95ºC for 2 min (initial denaturing); 40 cycles of 94ºC for 45 sec, 60ºC for 45 sec, and 72ºC for 45 sec (main reaction); and 72ºC for 10 min (final extension). Specific Forward and Reverse primers were designed to straddle the predicted insertion sites with theoretical melting temperatures between 55ºC and 60ºC. The sequences of primer pairs varied with each of the four *Xrn* genes and for each specific mutant insertion site, as well as a primer pair for control PCR to confirm amplification. See Table 2 for full list of primers, respective *Xrn* gene target, and predicted PCR product size from WT (non-transformed) control template. A Forward and Reverse primer pair (Exo3 and Exo9) that anneals to the 5' untranslated region (5' UTR) of *CrXrn1* gene, at a region not affected by a predicted insert, was used as a positive control for PCR. For each mutant strain, two independently isolated colonies from the same strain were analyzed due to the fact that some of the original strains might be mixed cultures with some cells having an insert and some without an insert[13].

Table 2. List of genomic PCR primers, Forward and Reverse, designed to amplify across the different insertion sites. The list includes proposed gene name, CLiP mutant strain number[15], designed Forward and Reverse primer sequences, predicted size (bp) of PCR product from WT genomic DNA (without insert), and predicted insertion site. Note, the predicted insertion sites in mutant 117676 and 105856 are sufficiently close within *CrXrn1*, Intron 12 and 13 respectively, that the same primer pair was used to analyze both mutants.

Proposed Gene Name	**CLiP Mutant Number[15]**	**Primers: Forward and Reverse**	**Size of PCR Product in WT**	**Predicted Insertion Site[15]**
CrXrn1	RY0402.**105856**	Forward: 5'-CCCACCTACTGCAAGTCC-3' Reverse: 5'-AGGCCAGTTGATGTAGGC-3'	654 bp	Intron 13
" "	RY0402.**117676**	" "	" "	Intron 12
CrXrn2	RY0402.**174346**	Forward: 5'-GTTCATGTGCTTCTTCTGC-3' Reverse: 5'-GTTTGCTGAAGATGATGTCC-3'	467 bp	Intron 9
CrXrn3	RY0402.**114070**	Forward: 5'-AGCAGTAGCAGCAACACG-3' Reverse: 5'-GCCATCTCAGATCGAACC-3'	484 bp	Intron 3
" "	RY0402.**171169**	Forward: 5'-GAGGGATTTGGAATGAGG-3' Reverse: 5'-TTGTGTCGCAGCATGG-3'	474 bp	Intron 4
" "	RY0402.**190466**	Forward: 5'-GCTGCAGCTGTTGTGC-3' Reverse: 5'-CTTCATCTTCGTCTTCTTCG-3'	556 bp	Intron 10
" "	RY0402.**197122**	Forward: 5'-CTGGGGCTGCAGAAGG-3' Reverse: 5'-CACGACCCCCATAACC-3'	494 bp	Intron 15
CrXrn4	RY0402.**042261**	Forward: 5'-GGTTAACGCTTCATGTTCC-3' Reverse: 5'-GACAGCGGTCTGGTAGG-3'	681 bp	Exon 9 / Intron 9
" "	RY0402.**078140**	Forward: 5'-AGTGTCTCTTTGCCAGTCC-3' Reverse: 5'-CCACTTAAGGGTGAGATGG-3'	479 bp	3' UTR, 5' of polyA site
" "	SG0182.**007713**	Forward: 5'-GTGTATGAGTCGCTGTACG-3' Reverse: 5'-GTCTACGGGCAGACTCC-3'	520 bp	Intron 12 / Exon 13
CrXrn1	+ Control	Exo3: 5'-GCACAAGATCATGGAGTACA-3' Exo9: 5'-CATCAGCTTGTTCAGCGCGC-3'	676 bp	5' UTR

PCR products were analyzed in agarose gels by mixing 15 µl of PCR amplified DNA with 2.0 µl of 10 X DNA loading buffer. DNA samples were loaded in a 1% agarose, 1 X TAE gel and electrophoresed at 90 – 100 volts for 30 – 45 min. Ethidium bromide (0.5 µg/ml) was used to stain DNA, and gels were visualized with Syngene's *Gene Genius Bios Imaging System* using *GeneSnap* software, version 7.07.01 (Syngene Corporation).

3. Results

Using gene sequence and locus identification numbers for the four predicted *C. reinhardtii Xrn* genes, as well as enzyme name, the CLiP mutant library website was searched to identified all possible putative mutants in the four predicted *Xrn* genes[9, 15]. These identified a total of ten different predicted mutants from the CLiP library (see Table 1). All ten strains were obtained and cultured.

To confirm the presence of the transformed insert DNA with *AphVIII* gene, which confers resistance to the antibiotic paromomycin, the ten *C. reinhardtii* mutant strains were tested for growth on TAP plates as well as TAP + paromomycin plates (Figure 1). All ten mutant strains plus WT control grew on TAP plates (without paromomycin), as expected. While only the ten mutant strains grew on TAP + paromomycin (20 µg/ml and 30 µg/ml) plates. As expected, the non-transformed WT strain did not grow on paromomycin-containing TAP media for it lacked the paromomycin resistance gene. These data confirm phenotypically the presence of the transformed insert DNA in each of the ten mutant strains.

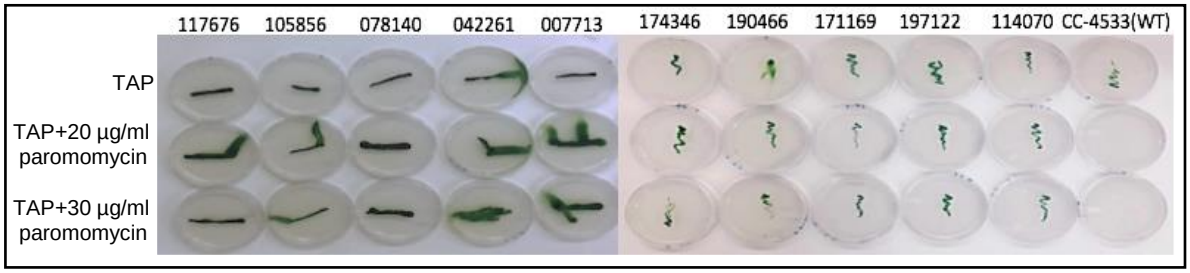

Figure 1. Growth of WT and mutants tested on paromomycin-containing TAP plates.

WT (CC-4533) and the ten *Xrn* mutant strains (see Table 1 for corresponding CLiP mutant numbers) were incubated on TAP and TAP + paromomycin (20 µg/ml and 30 µg/ml) agar plates for one week. This confirmed the paromomycin-resistant phenotype expected for strains that contain the transformed insert DNA. These data, however, are not able to assess the location of the insert within the *Xrn* genes of interest.

To test if transformed DNA was inserted into the predicted *Xrn* genes at the respective locations, based on the specific mutant strain, a preliminary PCR genetic screen was conducted. The initial genetic screen used PCR Forward and Reverse primer pairs designed to anneal to and straddle the specific *Xrn* gene sequence at the predicted 3-kb insert location, so as to amplify the DNA surrounding and including the insertion site. Thus, a WT strain (without the 3-kb insert) would amplify a predictable, small PCR product across the insert site. In contrast, amplification of genomic DNA from a mutant strain with the expected 3-kb insert would either not amplify, due to insert located between primer annealing sites and its large size, or would generate a PCR product ~3-kb larger due to insert. To control for variation in genomic DNA quality and assure PCR amplification from each genomic DNA sample, a PCR positive control was included that amplifies DNA from a non-insert site. Figure 2 presents representative data from two distinct colonies obtained from two different strains that demonstrate examples for both presence of the insert DNA (strain 007713, colony #2, with predicted mutation in *CrXrn4*) and absence of an insert at the predicted site (strain 114070, colonies #1 and #2). The latter strain still contains the insert, since it grew on paromomycin media (Figure 1), but at a different chromosomal location. Therefore, it is most likely not an *Xrn* mutant.

To date, nine of the ten mutants have been PCR analyzed, and of these, four (44%) have evidence consistent with predicted DNA insertion. These four CLiP mutant strains are 007713 (*CrXrn4* gene), 171169 (*CrXrn3* gene), 174346 (*CrXrn2* gene), and 197122 (*CrXrn3* gene). One of the nine, 117676 (*CrXrn1* gene), has shown varied results that indicate it might be a mixed culture, and we continue to analyze this strain. Four strains (105856, *CrXrn1*; 114070, *CrXrn3*; 190466, *CrXrn3*; and 042261, *CrXrn4*) appear to not have an insert at the expected location, and therefore, are likely not *Xrn* mutants. The analysis to date for the final, tenth, strain (078140, *CrXrn4*) has been inconclusive due to technical problems with PCR.

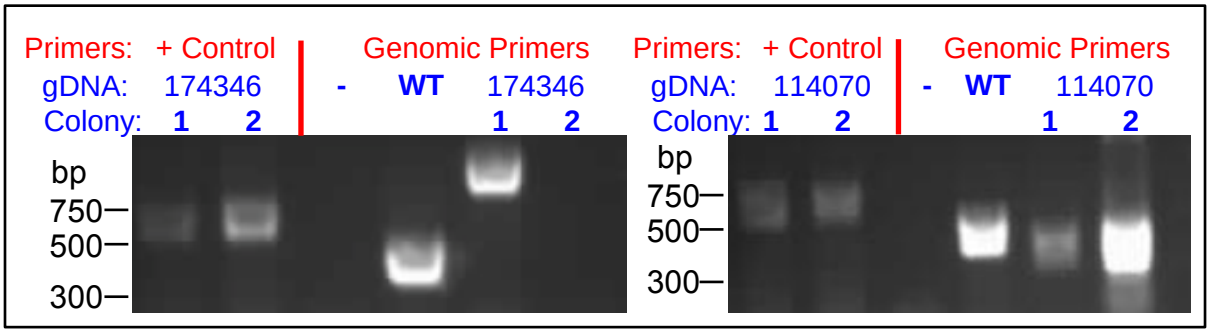

Figure 2. Representative agarose gel with DNA from initial PCR screen of mutants.

Total genomic DNA from two separate colonies for each of the mutant strains (1174346 and 114070) were PCR amplified using the + Control primer pair to demonstrate that the isolated DNA was competent for PCR. Next, the genomic primer pair specific to the gene and insertion site was used to amplify from a no DNA negative control ("-"), the WT genomic DNA positive control ("WT"), and DNA from two colonies from the mutant strain of interest. Strain 174346, colony #2 showed the absence of an amplified PCR product while the WT control (467 bp) and + Control (676 bp) both amplified the expected sized PCR products. These data are consistent with 174346, colony #2 being a *CrXrn4* mutant that has a DNA insert located within intron 9. As for strain 174346, colony #1, PCR with the genomic primers amplified a ~2-kb fragment, larger than WT fragment (467 bp), indicating it might also be a mutant but with a truncated insert, which has been shown to occur[13]. In contrast, colonies #1 and #2 from mutant strain 114070 both amplified WT-sized DNA fragments (484 bp) with the gene-specific genomic primers, suggesting that neither of these cultures contain an insert in *CrXrn3* at intron 3. Thus, CLiP strain 114070 appears to not contain a *CrXrn3* mutation, and the insert with paromomycin resistance gene must exist elsewhere in the genome.

Based on this preliminary PCR screen, four of the assessed nine mutant strains are likely *Xrn* mutants. To test for growth phenotypes of the *Xrn* mutants 7-day growth curves and diluted spot plating assays are being used. To date, two of the *Xrn* mutant strains (007713, in *CrXrn4*; 117676, in *CrXrn1*) have been tested (Figure 3).

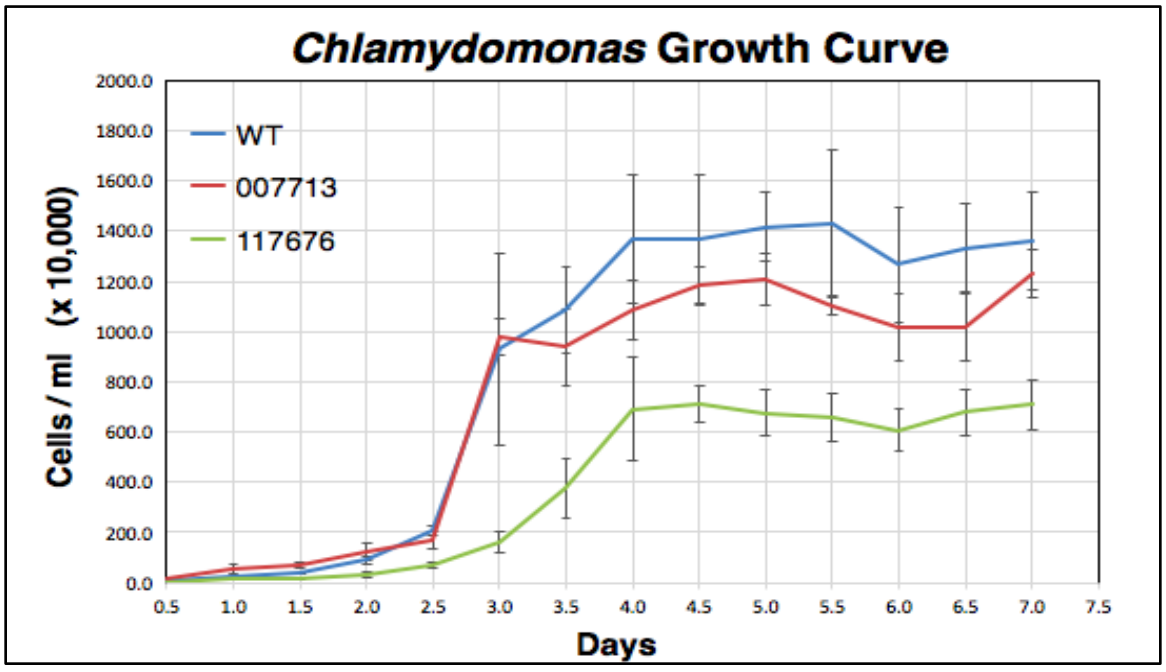

Figure 3. Growth curves to assess phenotypes of predicted mutants compared to WT.

Growth curves for strains 007713 and 117676 were compared to the WT control, and for each strain three independent replicate cultures were tested. Cell density (cells / ml) was determined every 12 hours over the 7-day growth curve. The average value from the three replicates is presented for each strain and each time point, with error bars indicating standard deviation. From these data, strain 117676 (with predicted mutation in *CrXrn1*) had consistently lower cell density, slower growth, and saturated at a lower cell density, as compared to both WT and 007713. In contrast, strain 007713 (with predicted mutation in *CrXrn4*) had a growth rate and cell density similar to WT. Although 007713 may appear to have slightly lower cell density over the last three days of culturing, these data are not significantly different from WT under these growth conditions.

To confirm cell density growth curve data, diluted spot plate assays were performed to independently evaluate growth over the same 7-day time course. For this, samples from two of the independent replicates for each strain were diluted and spot plated on TAP agar. These were incubated for five days to visualize amount of growth (Figure 4). Although spot plates from all seven days were obtained, after three days the cell density was sufficiently high in all cultures that differences between strains were not evident in these spot assays. Thus, only plates from the first three days, including the "Day 0" start, are shown.

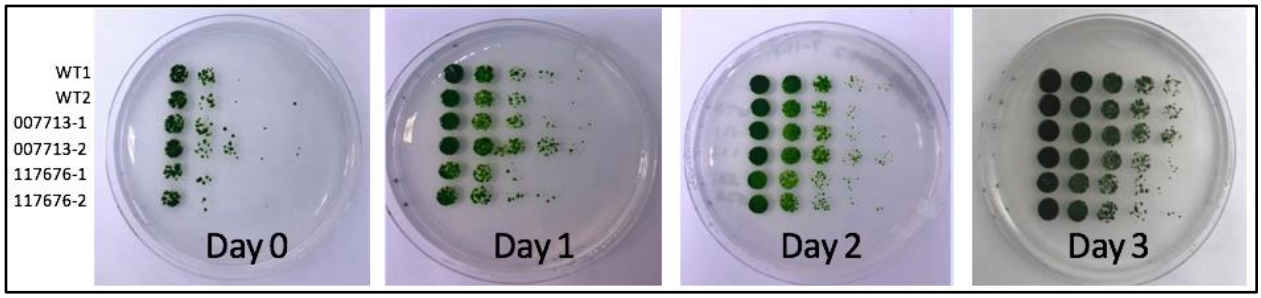

Figure 4. Diluted spot plate assay to independently test growth phenotypes of mutants compared to WT.

For the diluted spot plate assays (Figure 4) the dilution series, from left to right, for each culture is: "undiluted", "1/10", "1/100", "1/1,000", and "1/10,000". The "Day 0" plate from start of the 7-day growth curve shows that cell densities were generally the same across all replicates for all three strains, as expected. However, after three days, strain 117676 had about a ~10-fold lower cell density as compared to WT. This is most evident by comparing the amount of growth on the "1/100" diluted spots of strain 117676 to the growth on the "1/1,000" diluted spots of WT. These spot plate data are consistent with the cell density growth curve data (Figure 3).

4. Discussion

The ten *C. reinhardtii* CLiP mutants, predicted to have insert DNA in one of the four *Xrn* genes, have been tested phenotypically and using an initial PCR genetic screen. All ten CLiP mutants are resistant to paromomycin and grow on TAP + paromomycin (up to 30 µg/ml). This confirms that all ten have the transformed insert DNA with the *AphVIII* gene, as expected. The initial PCR screen was completed for nine of the ten mutants and tested for the presence of the insert DNA within an *Xrn* gene and at the predicted location. PCR data determined that four of the nine mutant strains (44%) appear to contain the insert DNA at predicted locations. These four *Xrn* mutant strains are 007713 (*CrXrn4* gene), 171169 (*CrXrn3* gene), 174346 (*CrXrn2* gene), and 197122 (*CrXrn3* gene). PCR analysis of the strain 117676 (*CrXrn1* gene) had varied results that indicated it might be a mixed culture with some cells containing the expected insert while others do not. A phenomenon previously reported for CLiP mutant strains[13]. We will continue to analyze this strain to resolve if indeed it is a *CrXrn1* mutant or not. The remaining four strains (105856, *CrXrn1*; 114070, *CrXrn3*; 190466, *CrXrn3*; and 042261, *CrXrn4*) appear to not have a DNA insert at the expected location and are therefore most likely not *Xrn* mutants. Finally, PCR analysis for the tenth strain (078140, *CrXrn4*) was inconclusive, and further analysis is needed to determine the status of DNA insert in this strain.

A 44% frequency of strains with predicted mutant insert is lower than the published 75% frequency[13]. The complex nature of random DNA inserts can complicate mapping the predicted location of these inserts. As previously reported, diverse and unpredicted events can occur when transforming insert DNA that can lead to two inserts present in one strain, two strains in one initial colony ("mixed culture"), additional endogenous *C. reinhardtii* genomic DNA fragments co-insert with transformed DNA that complicates insert mapping, deletion of genomic DNA during

insertion events, and various types of truncated insert DNA[13]. It is not clear if the reduced frequency (44%) presented here is specific to the *Xrn* mutants, which might suggest critical functions for *Xrn's*, or if instead the lower frequency is merely due to the small sample size of only nine analyzed strains.

To positively confirm the presence of the insert DNA, a second PCR screen will need to be conducted for those four strains that appear to contain an insert in an *Xrn* gene. This second PCR will use primers internal to the transformed insert DNA paired with genomic primers to generate detected PCR products that straddle the insert junctions, at 5' and 3' ends of the insert. These PCR products will be sequenced to positively confirm the location and presence within the specific *Xrn* gene.

With preliminary evidence from two of the mutant strains, initial growth curve and diluted spot plate assays were conducted. These were performed by growing mutants and WT under standard growth conditions. To date, growth curves and diluted spot plate assays have been performed for two mutants, 007713 (*CrXrn4* gene) and 117676 (*CrXrn1* gene), as compared to the WT. From these data, the 007713 strain does not appear to have an obvious change in growth compared to WT, under these conditions (Figures 3 and 4). To further evaluate 007713, additional tests will be conducted by growing it in environmentally stressful conditions, high/low light conditions, high/low temperature, and limited nutrients, to name a few. In contrast, 117676 does have a significantly reduced cell density and growth rate compared to both WT and 007713 strain under these growth conditions. This too will be further tested for more extreme growth phenotypes under environmentally stressful conditions. An ongoing concern about 117676 strain is the somewhat inconsistent PCR data that leads us to question whether it indeed has an insert at the predicted site and within the *CrXrn1* gene. This will make the second PCR screen critical to confirm whether the insert DNA is indeed in the predicted location in *CrXrn1*. Currently, we cannot rule out that the insert DNA is located in a different, non-*CrXrn1* gene, and this might be the cause for reduced growth.

Finally, confirmation of DNA insert locations in the *Xrn* genes by the second PCR screen is needed before attempts to test the molecular phenotypes of *Xrn* mutants are made. Determining molecular phenotypes would involve testing for changes in candidate target RNAs normally degraded by a given Xrn protein. Candidate RNAs would include cytosolic and organellar RNAs, such as the chloroplast *petD* mRNA. It is hypothesized that loss of an Xrn protein due to insertion mutations would cause an increased accumulation of target RNAs, which in turn may alter abundance or sequence of other RNAs or proteins. Based on known functions of Xrn proteins from different organisms (plants, animals, and fungi) a wide range of RNA types (mRNAs, rRNAs, miRNAs, etc..) could be affected[11, 17, 18, 19]. Ultimately, RNA-Seq could be used to assess the full transcriptome of *Xrn* mutants as a way to determine changes in RNAs. These would provide important new information about the roles and targets of these four *Xrn* genes in this important model alga.

5. Acknowledgements

The authors would like to thank The Scott and Alice Thomson Research Fellowship for funding to D.Y.C. and V.E.R.; the Dean of the College of Natural and Health Sciences at the University of Wisconsin-Parkside for research funds for V.E.R; and the University of Wisconsin-Parkside Undergraduate Research Apprenticeship Program for funding to D.Y.C. and V.E.R.

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