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Molecular Analysis of a *Chlamydomonas reinhardtii* Mutant That Tolerates Low Nitrogen

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Abstract

Organisms regulate the expression of genes in response to many factors, including developmental and environmental changes. Regulated RNA stability is one way to control gene expression. In eukaryotes, 5'-3' exoribonucleases (Xrns) help control mRNA stability that in turn can influence abundance of encoded proteins and regulate gene expression. Sequence analysis predicts four *Xrn* genes in the nuclear genome of the single celled green alga *Chlamydomonas reinhardtii*, a model organism commonly used to study photosynthesis. Two of the *Xrns* appear to encode Type I Xrn proteins, typically targeted to the cytoplasm, while the other two appear to encode Type II Xrn proteins, typically targeted to the nucleus. Ten mutant strains with a predicted DNA insertion into *Xrn* genes were obtained from the Indexed, Mapped Mutant Library in *C. reinhardtii*. Growth tests confirmed that the mutants contain a selectable antibiotic marker, and PCR and DNA sequencing has confirmed the location of the insertion within the *CrXrn* genes for three mutants. To test phenotypes of the mutant strains, growth curves were performed with nutrient rich (TAP) media and nutrient-limiting media such as nitrogen and phosphate starvation and lack of acetate, a carbon and energy source utilized for facultative heterotrophy. On nitrogen-depleted media, one of the mutants grows to a higher cell density compared to wild type, and it accumulates a lower chlorophyll content per cell. This is of interest because nitrogen is a critical and common limiting nutrient for plant growth, and an analogous genetic variant may be possible to engineer in crop plants so as to overcome this nitrogen limitation, potentially allowing for the reduction of nitrogen fertilization.

Keywords: *Chlamydomonas*, 5'-3' exoribonuclease, nitrogen

1. Introduction

5' to 3' exoribonucleases (Xrns) are found in eukaryotes and are important for RNA degradation, nucleotide availability, and RNA processing[1]. Xrns degrade RNA one nucleotide at a time from the 5' to 3' end and may target specific RNAs based on location[2]. Xrns also play an important role in ribosomal RNA processing when localized to the nucleus and appear to function as transcription termination factors. In addition to their functions of recycling nucleotides and RNA processing, Xrns are considered regulatory factors[3]. Xrns can control mRNA stability, thereby changing the relative abundance of encoded proteins. These regulatory processes can be in response to developmental and environmental changes[3].

Regulation of gene expression that affects RNA stability is important, and in plants and algae this helps control chloroplast functionality by nucleus-encoded proteins[4,5,6,7]. Four *CrXrn* genes are present in the single cellular green algae, *Chlamydomonas reinhardtii*. Two of these, *CrXrn1* and *CrXrn4*, are predicted to encode Type I Xrns, which are typically targeted to the cytoplasm, while the remaining two, *CrXrn2* and *CrXrn3*, are predicted to encode Type II Xrns, which are thought to be targeted to the nucleus[8]. These *CrXrns* might have roles in regulating mRNA, which encodes proteins expressed in the chloroplast of *C. reinhardtii*[5,6].

The specific aim of the work described here is to determine the exact sequences and the effects of predicted loss-of-function mutations in *Xrns* on *C. reinhardtii* gene expression and physiology. Ten mutant strains predicted to contain *CrXrn* insertion mutations were acquired from an indexed, mapped Chlamydomonas Library Project (CLiP)[9]. These strains were generated by transformation and random insertion of the CIB1 insert that includes the paromomycin resistance gene, as well as an insert-specific identifying sequence, (barcode) unique to each insert. If the CIB1 insert is transformed into a part of a gene required for function, we would expect a loss or reduction of function of the encoded product.

In previous work, the ten mutant strains with paromomycin resistance were screened by an initial PCR to assess if an insert was within the predicted *CrXrn*[10]. Three of the ten strains were confirmed to have an insertion within predicted *CrXrn* genes. One of these three mutants, 7-1 strain, was predicted to contain an insert in *CrXrn3*, a type 2 *CrXrn* thought to encode a protein localized to the nucleus, and this mutant allele was termed *CrXrn3-1*. In work presented here, the position and exact sequence of *CrXrn3-1* insertion was determined through PCR and DNA sequencing.

Previous experiments examining growth of 7-1 mutant under low nitrogen stress had preliminarily showed that 7-1 had higher growth and cell density after 7-days of culturing as compared to both WT and other *CrXrn* mutant strains[10]. Here we confirmed 7-1 indeed has a tolerance to low nitrogen (TLN) phenotype and grows to higher cell density in media containing 1, 10, and 25 percent of the standard nitrogen content of TAP media. Similar growth curves tested 7-1 in low phosphate media, however, there was no significant difference between the 7-1 and the WT, suggesting the 7-1 TLN phenotype is specific to nitrogen stress. Additionally, chlorophyll content per cell of the 7-1 mutant was observed to be significantly lower than the wild type in all media except for Min and N10 Min media. Preliminary data indicate no genetic linkage between the *CrXrn3-1* mutation and the TLN plus paralyzed flagellum phenotypes, suggesting that the TLN phenotype is instead due to a second, non-linked mutation elsewhere in the genome.

This tolerance to low nitrogen is of interest because bioavailable nitrogen is one of the most common limiting factors for plant growth. Furthermore, runoff of excess nitrogen fertilizer into bodies of water that border agricultural fields often causes algal blooms, resulting in ecological problems[11]. Depending on the exact nature and biological mechanism of this phenotype, it may be possible to engineer an analogous genetic variant that exhibits a similar tolerance to low nitrogen levels in crop plants such as corn, wheat, or rice. Having a low nitrogen tolerant crop variety could be valuable both economically and environmentally[12].

2. Materials and Methods

2.1. Algal Strains and Preparation of Media.

The Wild Type (WT) mating type$^-$ strain (CC-4533) is the recipient transformed with the CIB1 DNA while the 7-1 strain is the CLiP mutant strain number LMJ.RY0402.171169-1[9]. All *C. reinhardtii* strains were grown and maintained on TAP (Tris Acetate Phosphate) media. TAP media includes acetate and contains all nitrogen, phosphate, and trace elements needed for optimal growth of *Chlamydomonas*[13]. Variant versions of media used in growth curves included TA, N25, N10, N1, Min, N10-Min, and N1-Min. TA media was TAP media without phosphate while N25, N10, and N1 media were adjusted to contain 25, 10, and 1 percent of the total amount of nitrogen found in TAP media, respectively. Min, or minimal, media is equivalent to TAP without acetate. Likewise, N10-Min and N1-Min are derivations of media that contain no acetate and only 10 and 1 percent nitrogen compared to TAP, respectively.

2.2. Sequencing CIB1 Inserts

Ten milliliter aliquots of cells from the WT and 7-1 were taken at mid-log phase in 50 ml TAP media, centrifuged, and cell pellets were stored at -80^{o}C. From those samples, genomic DNA isolation was performed using a phenol-chloroform extract procedure[14], and the resulting pellet was resuspended in 1x TE. To confirm the presence and quality of genomic DNA, samples were electrophoresed in a 0.8% agarose gel. Isolated DNA was stored at -20^{o}C.

The location of the CIB1 insert into *CrXrn3* in strain 7-1 was initially predicted[9], but presence of the insert needed to be positively confirmed and detailed the nature of the sequence needed to be determined. For this, forward (F7-2: 5'- GAGGGATTTGGAATGAGG-3') and reverse (R7-2: 5'-CCATGCTGCGACACAA-3') primers to *C. reinhardtii* genomic DNA straddling the predicted CIB1 insert site were designed for PCR. In addition, internal primers to the CIB1 insert were used to amplify the 5' (5'- AGGCTTGACATGATTGGTGC-3') and 3' (5'-GACGTTACAGCACACCCTTG-3') ends of the insert for the 7-1 strain. PCR tubes containing the appropriate

primers were prepared and amplified using a Bio-Rad C1000 Thermal Cycler with the program: 30 cycles each with 94ºC, 45 sec; 50ºC, 45 sec; 72ºC 2 min). Both WT and 7-1 *CrXrn3* genomic DNA at the predicted insert site were amplified using the external forward and reverse genomic primers. To amplify the 5' end of the internal CIB1 insert at *CrXrn3-1* the forward genomic F7-2 and reverse CIB1 5' internal primers were used and to amplify the 3' end of the internal CIB1 insert at *CrXrn3-1* the forward internal CIB1 3' and reverse genomic R7-2 primers were used. PCR products were electrophoresed in 1.5% agarose, 1x TAE gels, and bands at the desired lengths were excised, and these were purified using a GeneClean procedure, according to the manufacturer's protocol (MP Biomedical). The purified PCR products were sent to the UW Madison sequencing facility. The *A Plasmid Editor*, or *ApE*, software was used to convert sequence reads into nucleotide sequences. The known conserved sequence of the CIB1 insert was provided by the indexed, mapped mutant library[1], and the *CrXrn3* sequence was sourced from Phytozome (*https://phytozome.jgi.doe.gov/pz/portal.html*). Reverse complements of each sequence were generated (*http://reverse-complement.com/)* and pairwise alignment was performed using the *EMBOSS Water Pairwise Sequence Alignment* (*https://www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html*).

2.3. Growth Curves and Chlorophyll Content

All growth curves used TAP media as a control, and the presented data at every time point are averages of three replicates for every combination of each strain in each type of media. Fifty milliliter cultures in 150 ml Erlenmeyer flasks were grown while shaking (110 rpm) in constant fluorescent light at 100 $\mu E/m^2/s$. Cultures were grown to 7 days with the option of extending the growth period to 9 days, depending on if the growth of the cultures had plateaued in response to reaching their carrying capacities in the media they were grown. This was most notably the case in growth curves that involved Min, N10-Min, and N1-Min media. Further, each of the growth curve experiment, with the exception of those involving N10 Min media, were repeated at least three independent times, and representative experiments are shown.

To initiate a growth curve, absorbance at 750 nm (OD750) of a stock culture was measured in triplicate by taking 1 ml samples. The average absorbance of the triplicates was used to calculate the volume to add to give an OD750 equivalent of 0.04, and this volume was added to new flask with 50 ml fresh media to start the growth curve. On each day following the start of the cultures at the same time (24 hours later), 1 ml samples were obtained after consistent hand mixing to resuspend cells, and the OD750 of each of the samples were measured. The OD750 accuracy of cell density measurements was confirmed by hemocytometer cell counts of each culture of each growth curve on the final day of growth, and Student t-tests were performed on final OD750 measurements and hemocytometer counts to test for significant differences between samples at a given time point.

To estimate total chlorophyll content, 1.5 ml samples were taken from a 50 ml culture and chlorophyll quantified as previously described[13]. Using a spectrophotometer, sample absorbances were measured at both 665 and 649 nm. The resulting values were then used in the equation (1) to calculate total chlorophyll concentration per sample in micrograms per milliliter.

$$\text{Total chlorophyll in } \mu g/ml = 6.10(A_{665}) + 20.04(A_{649}) \quad (1)$$

For each sample, the resulting chlorophyll concentration was then divided by its OD750 value to calculate the chlorophyll content per cell. The values of chlorophyll content per cell were later normalized in comparison to the average value of chlorophyll per cell of the WT on TAP media for each respective growth curve, with chlorophyll per cell of WT in TAP being treated at 100%. This allowed for the compilation of chlorophyll content data from all independently grown curves into one data set.

2.4. 7-1 Mutant x WT Genetic Crosses

The 7-1 mutant was tested for flagellar-based motility with a light microscope (Leica DM500) after induction of gametogenesis on low nitrogen N10 media prior to crossing[13]. Routinely, the 7-1 mutant showed roughly 1 – 5% of cells being motile (and 95 – 98% being non-motile but still having flagella), versus 90 – 100% motility of WT control cells, by microscopic inspection. The non-motile 7-1 cells still had flagella, as revealed by visual inspection under high contrast light microscopy (Leica DM500), consistent with paralyzed flagella phenotype[13]. To test for genetic linkage between the TLN phenotype, the *CrXrn3-1* mutation, and the paralyzed flagella phenotype, five different

crosses between the 7-1 mating type$^-$ strain and the WT mating type$^+$ strain (CC-5151) were performed[13]. The 7-1 was a very poor mater due to its paralyzed flagellum, and across all crosses very few zygotes were obtained. From the second cross only five progeny were obtained, two from one tetrad and three from another. Paromomycin resistance, TLN, and paralyzed flagella phenotypes were determined for progeny along with presence of *CrXrn3-1* insert by PCR screen, previously described. To test for the TLN phenotype, a growth curve in N10 media was performed, comparing OD750 of the progeny to the WT and 7-1 after 9 days. Additionally, the phenotypic expression of a paralyzed flagella was determined through visual observation by light microscopy, as described above.

To further test if the poor mating and paralyzed flagella phenotype were linked to TLN, five additional crosses with 7-1 mutant or the few TLN progeny from original 7-1 by WT crosses, described above, were performed. In all cases low cell motility (~1 – 5%) and low number of resulting zygotes were obtained from strains with TLN. As an attempt to increase mating efficiency different low nitrogen media were used to induce gametogenesis of 7-1, including the TAP-based N5 (5% nitrogen), N1 (1% nitrogen), and N0 (0% nitrogen) as well as a Soil Extract Media with no added nitrogen[13]. In addition, 10 mM dibutyryl-cAMP (SC-201567, Santa Cruz Biotechnology), which has been previously shown to increasing mating of some paralyzed flagella mutants, was used to treated 7-1 cells prior to mating[13]. After these different media and dibutyryl-cAMP treatments the 7-1 cells were tested for cell motility microscopically and mating efficiency by crossing with WT then looking for formation of the zygotes and the resulting reticulate pellicle layer[13].

3. Results

3.1. Sequences of *CrXrn3-1* Insertion Mutation in 7-1 Strain

Genomic DNA was isolated from 7-1 mutant strain, and PCR was used to amplify the 7-1 insertion at 5' and 3' ends of the CIB1 insert with the genomic forward and 5' CIB1 internal primer as well as the genomic reverse and 3' CIB1 internal primer. The resulting PCR products were gel analyzed, gel purified, and sequenced. The 7-1 mutant contains a 2,217-bp insertion of CIB1 within intron 4 of *CrXrn3* (Cre08.g373916) on chromosome 8, and the insert includes a complete copy of the paromomycin resistance gene. It was also found to contain an additional 15-bp sequence at the 5' end of CIB1 insert and a 2-bp deletion of intron 4 of *CrXrn3* gDNA near 3' end of the insert junction. This mutant allele was named "*CrXrn3-1*", as it is the first characterized mutant in *CrXrn3*.

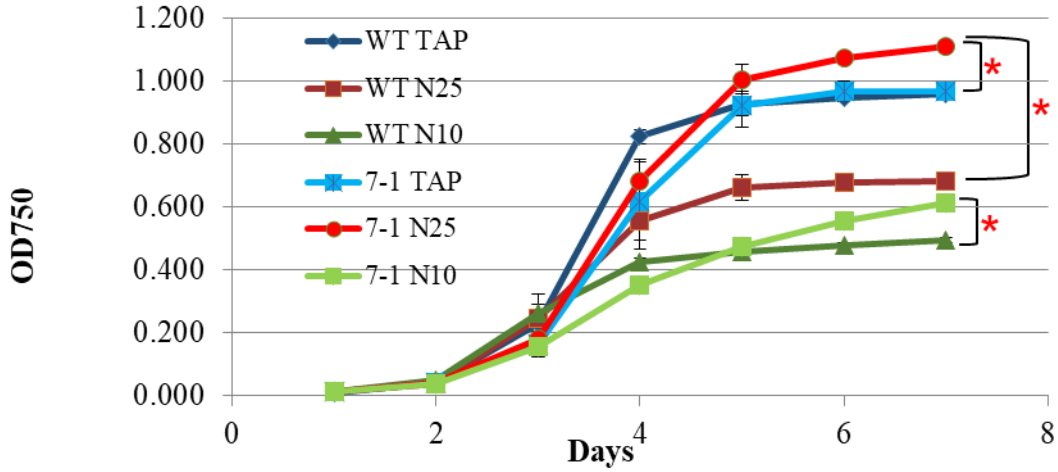
Figure 1. Diagram of genomic DNA *CrXrn3-1* mutant allele with CIB1 insertion that carries the paromomycin resistance gene. Exon 4 (open box), Intron 4 (black line), and Exon 5 (open box) are shown along with the 2,217-bp CIB1 insert (blue box) located near 3' end of intron 4. The large arrow indicates location and direction of transcription for paromomycin resistance gene. The small red box indicates the 15-bp additional sequence (GGCAAGCTAGAGAAC) within the 5' end of CIB1, and the small green box at 3' junction of CIB1 insert indicates the 2-bp (AC) deleted from Intron 4. A 500-bp size scale is shown.

3.2. WT Versus 7-1 Mutant Growth

From our previous work, it was known that the 7-1 mutant grew to a higher cell density in low nitrogen media than the wild type[10]. In addition, light microscopic observations (data not shown) revealed that 7-1 also has a paralyzed flagella phenotype as compared to the WT along with a reduced mating efficiency from crosses to mating type + WT strain. A link between these two phenotypes is not surprising, considering known paralyzed flagellum mutants have been reported to also cause poor mating efficiency[15]. To further investigate the TLN phenotype growth curves with were performed using OD750 to measure turbidity of cultures as an estimate of cell density. A 7-day growth curve of 7-1 and WT in TAP, N25, and N10 media confirms a previous preliminary observation that 7-1 grows significantly better in low nitrogen as compared to WT (Figure 2A). In addition to OD750 data, the cell density of these cultures

was independently and directly measured using a hemocytometer (Figure 2B), and the OD750 and hemocytometer estimates were consistent. More specifically, a significant increase in OD750 of 62% was measured in the 7-1 compared to the WT when grown on N25 media, which is a much larger difference in proportion to those seen on N10. Interestingly, the OD750 of the 7-1 on N25 was significantly higher than the values for both the WT and 7-1 even when grown on TAP. The OD750 and hemocytometer data shown are representative of three independent growth curves, each with three replicate cultures of TAP, N25, and N10 media, and similar results were seen across all independent experiments. It is worth noting that the higher cell density in low nitrogen (N10 and N25) for 7-1 compared WT does not appear until between day 4 or 5. This is likely because it is not until then that the nitrogen becomes limiting for growth, and the overall slower growth in N10 takes longer to deplete the nitrogen such that a difference is observed.

A)



B)

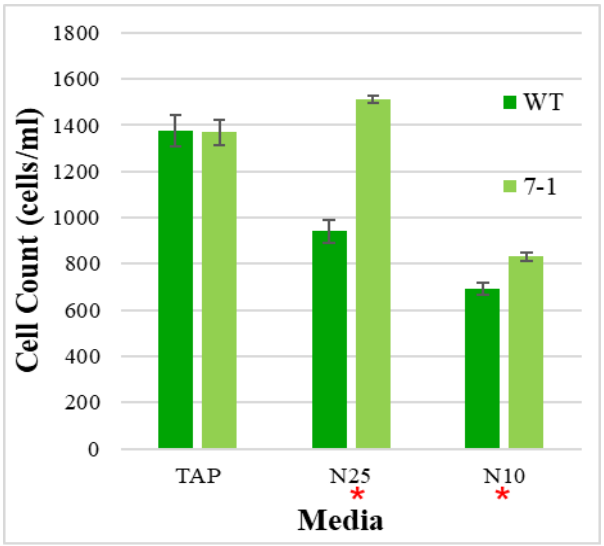


Figure 2. 7-day growth curve of WT and 7-1 in TAP, N25 and N10 media (50 mL). A) Cell density estimated by OD570. WT in TAP (dark blue) and 7-1 in TAP (light blue), $p>0.05$; WT in N25 (dark red) and 7-1 in N25 (light red), $p<0.0001$; WT in N10 (dark green) and 7-1 in N10 (light green); $p=0.002$. Datum points are averages of three replicates for treatment, and error bars represent standard deviation. B) Cell density in cells/ml as determined via cell counts using a hemocytometer. WT (dark green) in TAP and 7-1 (light green) in TAP, $p>0.05$; WT in N25 and 7-1 in N25,

p<0.0001; WT in N10 and 7-1 in N10; p=0.0015. Datum points are averages of three replicates for each treatment, and error bars represent standard deviation.

In order to distinguish whether the TLN phenotype is linked to photosynthesis or acetate metabolism, strains were tested over 9 days in TAP, Min (without acetate, phototrophic growth), N10, and N10-Min (phototrophic growth in low nitrogen) media (Figure 3). As seen previously, no significant difference (p>0.05) between the WT and 7-1 was seen in TAP, while a significant difference (p=0.0062) between the WT and 7-1 in N10 was observed. No significant differences between the WT and 7-1 were seen in Min or N10-Min media, even after 9 days. Notably, growth for both WT and 7-1 cultures had not plateaued, due to the slow growth, even after 9 days. Thus, in the slower phototrophic growth in Min media, neither WT nor 7-1 strains had reached a cell density that depleted the media of nitrogen such that nitrogen was limiting, as previously observed (Figure 3). To increase the nitrogen stress even more so as to reach a nitrogen limitation, 7-1 and WT strains were grown in N1 (99% reduction in bioavailable nitrogen), and N1-Min media (Figure 4). There was a reproducible higher cell density for 7-1 in N1-Min versus WT. However, these differences were not significant, and the p value was 0.057.

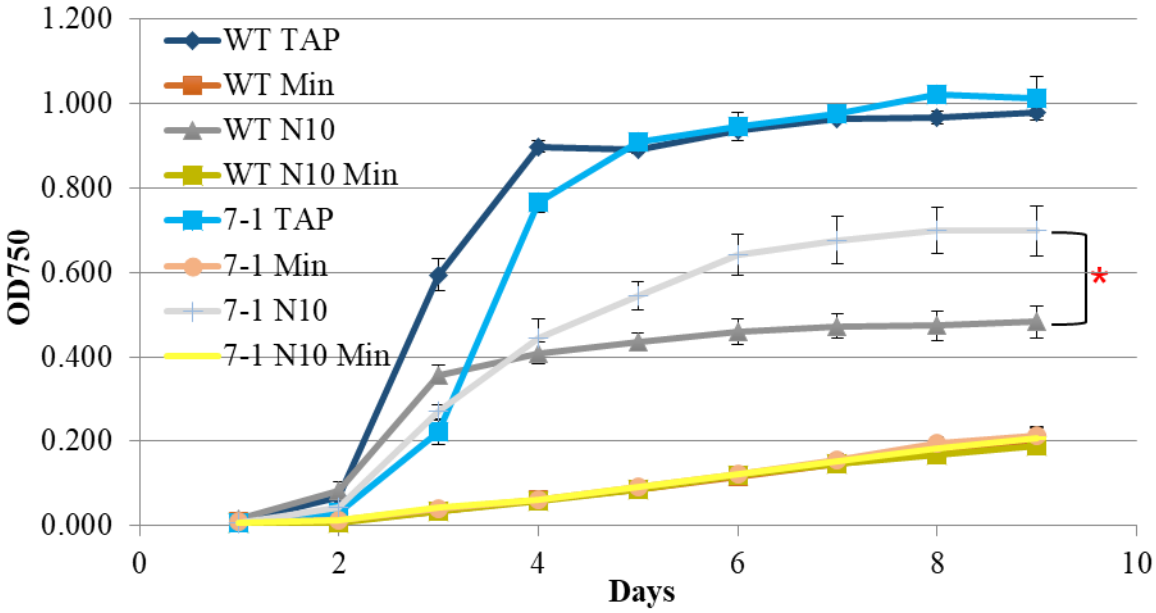


Figure 3. 9-day growth curve WT versus 7-1 in TAP, Min, N10, and N10-Min media (50 mL). WT in TAP (dark blue) and 7-1 in TAP (light blue), p>0.05; WT in N10 (dark grey) and 7-1 in N10 (light grey), p=0.0062; WT in Min (dark orange) and 7-1 in Min (light orange); p>0.05; WT in N10-Min (dark yellow) and 7-1 in N10-Min (light yellow); p>0.05. Datum points are averages of three replicates for treatment, and error bars represent standard deviation.

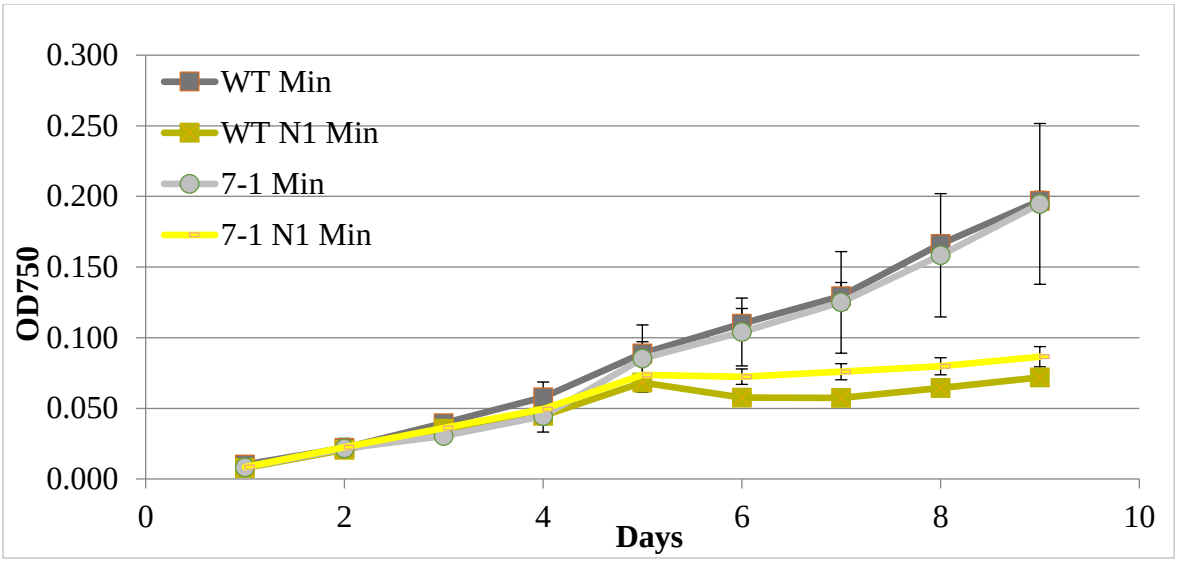


Figure 4. 9-day growth curve WT versus 7-1 in Min and N1 Min media (50 mL). WT in Min (dark grey), 7-1 in Min (light grey), WT in N1 Min (dark yellow), and 7-1 in N1-Min (light yellow). The difference in 7-1 versus WT in N1-Min is borderline significant (p=0.057). Datum points are averages of three replicates, and error bars represent standard deviation.

The higher growth of 7-1 versus WT in low nitrogen stress might be specific to nitrogen, or it is possible, there is an analogous increased in growth for 7-1 versus WT under different types of nutrient stress, such as low phosphate. To test this, growth curves comparing 7-1 and WT in both TAP and TA media (no phosphate) were performed (data not shown). Both the WT and 7-1 strain had significantly lower growth in TA media compared to TAP ($p < 0.05$), but no significant difference ($p > 0.05$) between the WT and 7-1 was seen in TAP, as expected. However, there was also no significant difference between the WT and 7-1 in TA media.

3.3. Chlorophyll Quantification

Despite higher cell density for 7-1 under low nitrogen (Figure 2), this strain did not appear significantly greener than WT by visual observation (data not shown). Chlorophyll a and b molecules are nitrogen rich, and they accumulate to high levels in algae. Furthermore, a significant change in the metabolism of this high-nitrogen molecule might help contribute to the tolerance to low nitrogen phenotype of 7-1. To test if 7-1 has altered chlorophyll abundance as compared to WT, a 7-day growth curve was conducted and samples were tested for total chlorophyll content[13]. Total chlorophyll concentrations (µg/ml) was determined, and this was divided by the OD750 to estimate the per cell chlorophyll concentration. These data were then compiled across multiple, independent growth experiments in a summative graph (Figure 5). A significantly lower chlorophyll content per cell was observed in the 7-1 compared to WT in TAP, TA, N25, N10, N1 and N1 Min ($p=<0.0001$, 0.015, 0.016, <0.0001, <0.0001, and 0.0013 respectively). However, there was not a significant difference between 7-1 and WT when grown in Min and N10 Min media ($p=0.93$ and 0.73 respectively. Thus, the cell density differences (Figures 2 – 4) do not correlate absolutely with chlorophyll content (Figure 5).

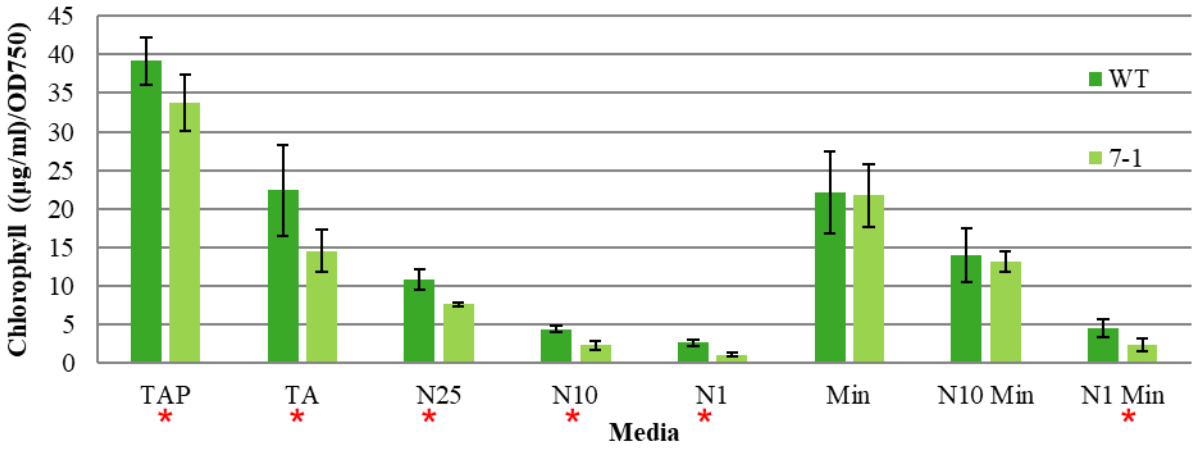


Figure 5. Chlorophyll per cell of the WT versus the 7-1 mutant across media types. Dark green is WT, and light green is 7-1 mutant. Data are averages obtained from between one and six independent experiments, each with three replicate samples for each treatment. (From left to right, p=<0.0001, 0.015, 0.016, <0.0001, <0.0001, 0.93, 0.73, 0.0013) Error bars represent standard deviation.

3.4. 7-1 x WT Progeny Studies

To determine if the TLN and paralyzed flagella phenotypes were caused by the *CrXrn3-1* insertion mutation, five different genetic crosses of the 7-1 strain with WT were attempted. The 7-1 mutant mated at a very low efficiency and results in a very low number of zygotes. Further, 7-1 cells have low cell motility (~1 – 5% cells were motile) after induction of gametogenesis, as determined microscopically after 3-day incubation on N10. In contrast, WT cells had 90 – 100% cell motility. To try to improve mating efficiency different media (N5, N1, N0, and Soil Extract Media) as well as a treatment with 10 mM dibutyryl-cAMP were used to improving mating efficiency[13]. In all cases, the 7-1 cells had low cell motility (~1 – 5% motile) and poor mating efficiency.

A total of only five progeny from the 7-1 x WT were obtained and analyzed for phenotypes (TLN, paralyzed flagella, and paromomycin resistance) and presence of *CrXrn3-1* insert by PCR (Table 1). From these preliminary genetic data, it is clear that the *CrXrn3-1* insertion is not genetically linked to the TLN and paralyzed flagella phenotypes. Specifically, four of the five progeny demonstrated a lack of segregation of the *CrXrn3-1* insert from the TLN and paralyzed flagella phenotypes (1-1, 1-3, 9-1, and 9-2). Thus, the *CrXrn3-1* insertion mutation is not the genetic cause of the TLN and paralyzed flagella phenotype, meaning these phenotypes are due to an unknown and unlinked inheritable mutation that has yet to be determined.

Table 1. WT x 7-1 strain progeny tested for genetic linkage of TLN and the *CrXrn3-1* mutation. The "+" symbol indicates the phenotype or CIB1 insertion (*CrXrn3-1* allele), while "–" symbols indicate an absence. **CrXrn3-1* insertion was tested by both PCR and paromomycin resistance. **TLN phenotype was tested by growth curve in N10 media. ***Paralyzed flagella was tested by light microscopy.

Strain	***CrXr3-1* Insert***	**TLN****	**Paralyzed Flagella*****
WT Parent	-	-	-
7-1 Parent	+	+	+
1-1 Progeny	+	-	-
1-2 Progeny	+	+	+
1-3 Progeny	-	+	+
9-1 Progeny	+	-	-
9-2 Progeny	-	+	+

4. Discussion

A 2,217-bp insertion of the CIB1 sequence was found within intron 4 of *CrXrn3* in the 7-1 mutant strain, and it contained a complete functional copy of the paromomycin resistance gene. This was a near-complete insert, but had a 15-bp sequence near the 5' end of CIB1 and a 2-bp deletion of *CrXrn3* gDNA at the 3' end of the insert junction. This large insertion may cause an inactivation of the *CrXrn3* gene and block the encoded protein, but this has yet to be confirmed through RNA and protein analysis. The 7-1 strain is paromomycin resistant, indicating that the inserted CIB1 DNA is functional within the middle of the *CrXrn3* gene. This may or may not affect expression and function of CrXrn3 protein.

The 7-1 strain with *CrXrn3-1* mutation grows to a higher cell density in nitrogen-deprived media, as demonstrated by growth curves on TAP, N25, and N10 media (Figures 2 and 3). One phenotypic observation that was surprising and potentially of great interest was the drastic increase (62%) in growth of 7-1 strain in N25 media (75% depletion of nitrogen) above both 7-1 and WT in TAP. When WT and 7-1 were tested in TA media (lacking phosphate), both strains grew at the same rate and to the same cell densities in both TAP and TA media. This indicates that the TLN phenotype is not a general nutrient stress response when any of the macronutrients are limiting, but instead likely specific to nitrogen stress.

The TLN phenotype appears to be specific to low nitrogen and shows a non-linear growth in response to bioavailable nitrogen. This suggests the TLN phenotype is somehow activated in response to nitrogen stress and in a way that the 7-1 strain responds differently than WT. This could be due to a gain-of-function mutation that alters cellular nitrogen metabolism, or its regulation, to allow higher-than WT growth when under depleted nitrogen. Alternatively, it could be due to a loss-of-function mutation that blocks some gene regulation or metabolic process. An additional possibility might be an increased nitrogen uptake and assimilation. There are eight known high and low affinity ammonium transport proteins (AMT) in *C. reinhardtii*[16,17]. The known mutants for these genes results in a reduced growth and nitrogen uptake, but we cannot rule out the possibility that the 7-1 strain harbors a novel mutation in one of these *AMT* genes that increases nitrogen uptake.

Across the media types, except for Min and N10 Min media, a significantly lower amount of chlorophyll per cell in the 7-1 versus the WT was observed. Specifically, a higher cell density in the 7-1 on low nitrogen media was seen to correlate with decreased chlorophyll content per cell. However, significantly lower chlorophyll per cell was seen even when grown in TAP and TA media, which do not have any low nitrogen stress. This indicates that the correlation between the TLN phenotype and lower chlorophyll per cell is not a direct cause-and-effect relationship. Rather, it is possibly a downstream effect related to the TLN phenotype, for which the exact cellular or biochemical mechanism remains unknown.

To test for genetic linkage between the *CrXrn3-1* insertion (and paromomycin resistance) and TLN phenotype, the 7-1 and the WT strains were crossed, and the few resulting progeny were analyzed for TLN phenotype and presence of *CrXrn3-1* mutant allele. Contrary to expectations, the *CrXrn3-1* allele is not linked to the TLN or paralyzed flagella phenotypes. Although there are relatively few progeny due to this poor mating efficiency of the 7-1 strain, four of the five progeny show clear non-segregation of the insertion and these phenotypes. These data strongly suggest that the

TLN phenotype is due to another mutation at a separate location in the 7-1 mutant. The nature and origin of the TLN-causing mutation is unknown at this time, and it is unlikely to contain a complete CIB1 insertion due to the non-linkage between the paromomycin resistance phenotype and TLN phenotype. The presented data, however, show that the paralyzed flagella phenotype is directly linked to the TLN phenotype. This suggests that whatever mutation generated the TLN phenotype is also the cause of flagella paralysis.

5. Acknowledgements

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6. References

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