UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

USING CHLAMYDOMONAS TO IDENTIFY FACTORS INVOLVED IN ACCLIMATION TO LIMITED IRON

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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ABSTRACT

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The means for populations of organisms to be able to cope with changes to survive and reproduce have been demonstrated for many years through the various mechanisms of evolution. Organisms must acclimate to changes to survive and reproduce. Acclimation is an adjustment or response of an organism to its environment. *Chlamydomonas* a unicellular green alga is a good model organism to study acclimation. Acclimation was studied by limiting the availability of an essential nutrient iron, causing stress. Specifically, a screening method was developed to study the growth rate of wild type and mutated Chlamydomonas under normal and stressed conditions. This allows for a more focused understanding of the genes involved in acclimation. Mutants are tested in the screen, amplified if its result shows it is involvement in acclimation. The mutant is then identified to test its physiological and cellular functions.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank my advisor, Tony Sanderfoot along with my committee members for all their advice the past few years. I would to thank my family and friends for supporting me in everything I do.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

FIGURE PAGE

INTRODUCTION

The means for populations of organisms to be able to cope with changes to survive and reproduce have been demonstrated for many years through the various mechanisms of evolution. Through the process of evolution populations achieve both enhanced diversity and adaptation. Diversity represents variation within the population (e.g., phenotypic similarities and differences) from these original traits found in their common ancestor. Adaptations are inherited characteristics that enhance survival and reproduction (Reece et al., 2011). However, evolutionary processes often take a long time to occur, and populations face many short-term stresses that must be handled with more immediate tools at the individual level such as acclimation or compensation. Acclimation is defined as adjustment or response of an organism to its environment (Mazess, n.d, Leroi et al., 1994, Ledford et al., 2007). Compensation is a short-term change in physiology or behavior (increase in an activity to make up for the decrease in another activity) of an individual to increase survival for a short time frame (Reece et al., 2011). Although acclimation is a short-term response to environmental changes, the ability to usefully show acclimation or compensation can persist from several generations allowing for the study of genes involved that contribute to being acclimated (Leroi et al., 1994).

Common stresses organisms/cells face are changes in pH, temperature, water, and nutrient levels. Cells respond to stress using a variety of tools/mechanisms such as genetic differences, epigenetics, cell signaling pathways, enzymatic activity. (Bibbin et

al., 2017, Connorton et al., 2017). Although organisms face many different kinds of stresses, the focus of this study was on stressing organisms for nutrients that are essential.

Nutrient availability is important for proper development. Each organism has a particular amount of nutrients it needs for survival (*figure 1*). Too much or too little of certain essential nutrients can be toxic and detrimental to an organism. Nutrient availability in each niche (the relationship between an organism and its environment), contributes to variation in metal content, pH, and oxygenation of the habitat (Taiz et al., 2018).

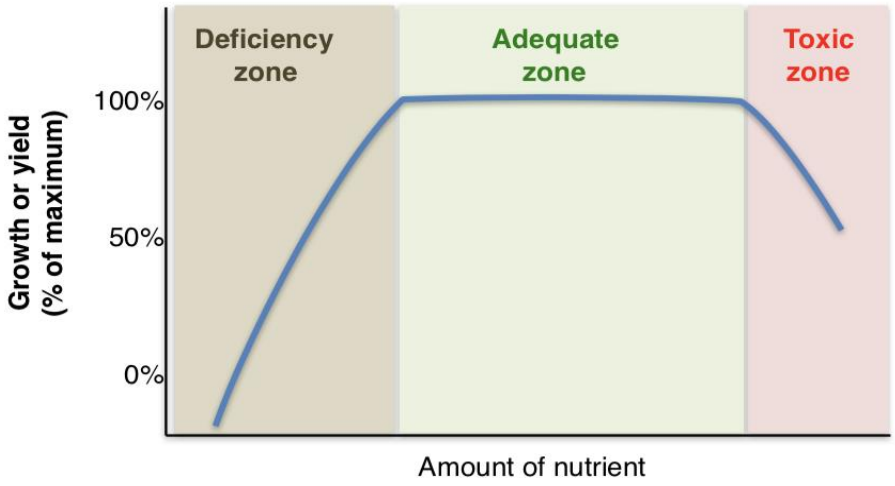

Figure 1. Nutrient amount versus ideal growth conditions of plants. All plants have an adequate/ideal zone for optimal growth. Modified image from Taiz et al., (2018).

The genetic model system I have used to study acclimation is *Chlamydomonas rheinhardtii* (Chlamydomonas), a single cell green alga. Chlamydomonas are found world-wide, occupy various environmental niches, and use many different nutrients to develop and survive (Merchant et al., 2006). Chlamydomonas are a simple model system that is readily and extensively studied (Li et al., 2019). The ability to successfully compete with the large variety of other organisms across many niches suggests that the

genus must have significant capacity for adapting to variations in nutrient availability (Merchant et al., 2006, Leroi et al., 1994). Survival in different nutrient amounts and environments also suggest a capability to show acclimation. Alterations of nutrients are known to cause changes in biochemistry and gene expression in Chlamydomonas (Lee et al., 2012, Ledford et al., 2007). The genome has already been sequenced allowing identification of potential genes that are involved in acclimation (Merchant et al., 2006).

Organisms rely on metal cofactors to catalyze many reactions that are essential to life (Blaby-Haas & Merhcant, 2012, Merchant et al., 2006). Iron is a component of many other proteins involved in reactive oxygen detoxification, fatty acid metabolism, mitochondria electron transfer and amino acid biosynthesis. Regulation of iron is important because too much or too little iron can cause cell damage (Bibbin et al., 2017, Connorton et al., 2017). Specifically, too much iron can generate reactive oxygen species which damage DNA (Glaesener et al., 2013). Iron is an important resource to Chlamydomonas because, like many plants and algae, they have two organelles with very high iron requirements: chloroplasts and mitochondria. The proteins within these organelles have essential functions in electron transfer pathways of bioenergetic membranes. Inadequate access to iron often limits photosynthesis due to dependence for trace iron elements for electron transfer (Glaesener et al., 2013). When photosynthesis is limited, Chlamydomonas has a yellow appearance (Harris, 2009).

A few specific genes in plants are known to have iron involved in their encoded function: *FOX1*, *FTR1*, *ACP1*, and *ADH1*. These genes should be affected by the limitation of iron. Metal transporters are important in the uptake and regulation of iron. The main uptake pathway in Chlamydomonas is a fungal-like ferroxidase dependent

ferric transport complex consisting of FOX1 and FTR1. FOX1 is the ferroxidase which catalyzes the oxidation of Fe (II) and Fe (III). FOX1 forms a complex with FTR, a ferritin that works as the permease to transfer the ferric iron into the cytosol (Glaesener et al., 2013, Morrissey & Guerinot 2009). ACP1 is an acyl carrier protein found in the chloroplast and involved in fatty acid synthesis (essential for membranes and energy storage) (Van Vranken et al. 2016). The iron sulfur cluster is an important biosynthetic process within the mitochondria. ACP plays a role in synthesis by keeping the iron sulfur cluster stable (Van Vranken et al. 2016). ADH1is alcohol/acetaldehyde dehydrogenase and plays an important role in anaerobic metabolism of photosynthetic unicellular organisms. ADH1 has metal ions, iron and zinc, in the active site (van Lis et al., 2017).

Acclimation is an organism's ability to adjust or respond to its environment that is essential for dealing with short term stress, such as limitation of an amount of an essential nutrient. Chlamydomonas is a good model organism to study acclimation because of all the various environments and stress conditions it survives in. A screen examining recovery of Chlamydomonas from a limited amount of iron was used to identify genetic factors involved in iron acclimation.

OBJECTIVES

1. **Examine acclimation of Chlamydomonas from essential nutrient stresses.**

I hypothesize that Chlamydomonas will acclimate to limited amounts of iron.

- Develop a screen that will show stress from lack of iron and find a condition in which wildtype Chlamydomonas recovers, showing identification of acclimation, a measurable phenotype.

2. **Examine genetic factors involved in acclimation of Chlamydomonas.**

I hypothesize that the ability of Chlamydomonas to show acclimation will have genetic factors that can be screened for.

- Use insertional mutagenesis and electroporation to screen for mutants that fail to recover from iron-starvation like the wildtype and identify the gene responsible.
- Put ordered mutations FOX1, FTR1, ACP1, ADH1 through screen to test for iron recovery.

3. **Understand further function of genes identified in objective 2 of Chlamydomonas; physiological aspects, cellular function, further application.**

I hypothesize that acclimation will alter the physiology in a way that can be measured.

- Generally, characterize the physiological aspects of genes in mutants from objective 2 through growth, photosynthesis, and respiration rates.
- Apply potential further large-scale application of results.

METHODS

Acclimation (Recovery) of Chlamydomonas from Essential Nutrient Stresses.

A screen was developed to study acclimation of Chlamydomonas from limited amounts of iron by determining if and how Chlamydomonas recovered from the limited amount of iron. This allowed for the understanding of how long/far cells can be stressed and still recover.

Chlamydomonas strains CC-406 or CC-5325 (acquired from the Chlamydomonas Stock Center) were grown in either standard liquid or on solid media. Tris Acetate Phosphate (TAP) medium is a mixture of the necessary macro- and micronutrients required by Chlamydomonas, including chelated iron, which allows for optimal growth and maintenance (Harris, 2009). To study acclimation, elements of the medium were eliminated, specially iron. TAP medium made it relatively easy to manipulate the concertation of the iron ion by selectively controlling it through eliminating it from the micronutrient solution.

Two mixtures of TAP were made one with all the essential nutrients needed for optional growth (normal) and one with all essential nutrients except iron (minus iron). Chlamydomonas was grown in a 1.5 mL tube of normal TAP for one day. All equipment was kept sterile to ensure no other elements were affecting the growth but limited iron. The concentration of Chlamydomonas cells was determined by a spectrophotometer to

ensure that the same amount of cells were added to each well. Cells were grown for one day in normal TAP then centrifuged to a pellet. The normal TAP mix was removed and a specific amount of TAP minus iron (dependent on the number of cells in the sample) was added so all the samples had the same number of cells. The samples were then left to sit for approximately 15 minutes before being added into a welled plate. Wild type and mutated Chlamydomonas were added to three replicate wells of either normal TAP, TAP minus iron, and TAP minus iron that will eventually have iron added back (plus iron), allowing analysis of recovery. When stress of Chlamydomonas was identified by observation of color change, a solution containing the required Fe-ions was added back to replicated samples and the recovery of the wildtype was observed by color change. The concentration of iron added back was based off the normal concentration of normal TAP Fe-ions in normal TAP solutions. This was repeated multiple times. The growth was measured multiple times (1-2 times a day) across a 5-7 day growth period by a spectrophotometer at 680nm (measure the chlorophyll absorbance) and 750nm (measures total absorbance, equivalent to total cells), as well as direct observations for physical color change (green versus yellow).

Examine Genetic Factors Involved in Recovery of Chlamydomonas.

To understand the genetics involved in acclimation both a non-targeted and targeted approach were taken to identify genes that may be involved in acclimation to limited amounts of iron in Chlamydomonas. In the non-targeted approach genes were disrupted by insertional mutagenesis of a marker trait using either chemical transformation or electroporation. Chemical transformation, inserting a known trait-marker (antibiotic resistance) into locations of the genome, created a mutation following

a modified method of Sizova et al. (2001). A fragment of DNA with a known sequence and a known selectable trait was randomly inserted into the genome: resistance to paromomycin (PAR) or hygromycin (HYG) (see *figure 2*). PAR or HYG DNA fragments were made by PCR following a modified method of Sizova et al. (2001).

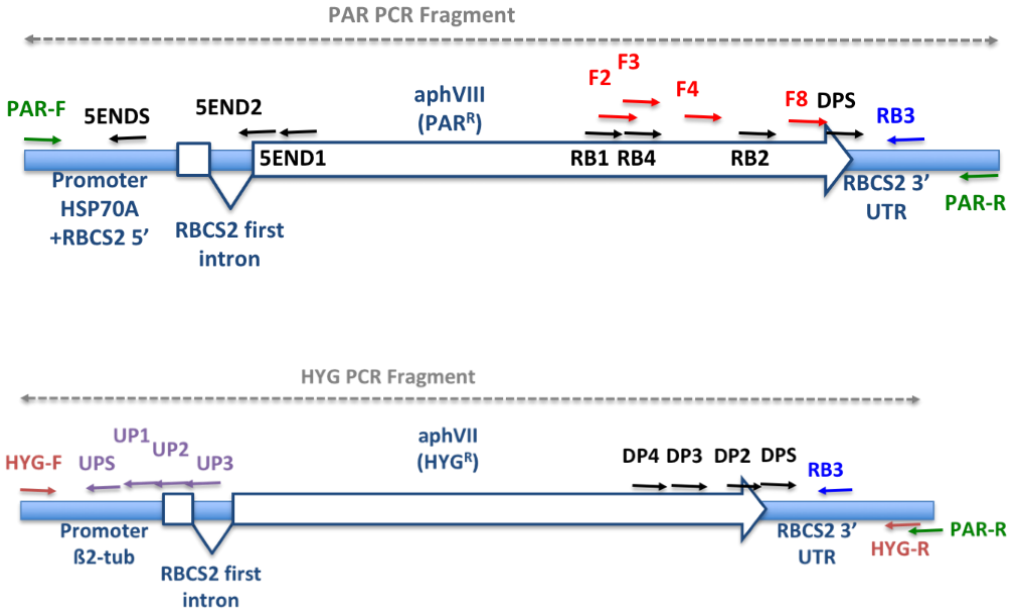

Figure 2. HYG and PAR double-stranded DNA fragments generated by PCR are shown by the blue boxes, with the white arrows representing the exons encoding the enzymes that result in resistance to the antibiotics. The small arrows above the boxes represent the name, location and direction of the primers used in the study.

Chemical transformation was modified from the method of Sizova et al. (2001). Briefly, wildtype Chlamydomonas were grown on a shaker for 4-6 days, then counted in a hemocytometer to have a transfer volume of culture that contained approximately $3x10^7$ cells in a 15mL tube. The culture was then centrifuged, and the liquid was removed, and the pellet was resuspended into new normal TAP. Glass beads and Polyethylene glycol (PEG) were added in drops to help break open the cell and allow the double-stranded

DNA (carrying resistance to paromomycin, PAR, or hygromycin, HYG, under control of Chlamydomonas regulatory regions) to be inserted into the genome. The culture was removed from the beads, centrifuged and provided fresh TAP to grow until the following day. The next day the mutants were plated on appropriate antibiotic plates for selection and left to grow.

Once the mutants grew for about a week the screen was repeated to identify if these mutants are involved in iron acclimation, as described above. The screen was done in a welled plate, three wells for each medium (normal and minus iron) containing either wildtype or the mutant. If the mutant is involved in acclimation, the color of the Chlamydomonas indicating growth will be less green (yellow) compared to the wildtype and will not return to the same level of greenness as the wildtype even when iron is added back. Recovery can also be shown by a lower spectrophotometer (e.g., a decrease of ~0.5 absorbance units [AU]) absorbance than the wildtype.

The second form of insertional mutagenesis was electroporation which is using electrical pulses to create pores in the plasma membrane, allowing entry of macromolecules (e.g., HYG or PAR DNA) into the cells. This method was modified from Molino et al. (2018). This process starts with Chlamydomonas grown on a shaker for 4-6 days, then counted in a hemocytometer and a volume of culture that contained approximately 200 million cells was transferred. The culture was then centrifuged, and the liquid was removed so the cells could be resuspended into cold TAP plus 40 mM sucrose (250µL at $2x10^6$ cells per mL). The cells were chilled for 10 minutes. The cells and ~150 ng of DNA was added to an electroporation cuvette, then placed in the BioRad electroporator (kindly provided by the Schwann lab). Initial studies tried various pulse

strengths, to find the most efficient settings (800 V, ∞ resistance, 25 μF capacitance having the greatest yield of transformed cells). Immediately, the cells were poured into 10 mL TAP plus 40 mM sucrose and allowed to grow for 6 hours and then the mutants were plated on appropriate antibiotics for selection and left to grow (Molino et al., 2018).

Restriction site directed polymerase chain reaction (RESD – PCR, *figure 3*) was done to identify insertion sites of known DNA in the mutant (modified from Gonzalez-Ballester et al., 2005). Identification of an insertion site in a mutant is difficult due to the large genome and high G/C-base content of Chlamydomonas. Two-to-three rounds of RESD – PCR that use nested primers and less degenerate primers (see arrows in *figure 2*) are required. RESD – PCR has longer primers that have reduced degeneracy but have sequences that are statistically over – represented and are binding sites for reaction enzymes. These enzymes recognize a sequence of DNA and make a double strand break. There are certain 4-6 base pair restriction sites that are more or less common in DNA. In Chlamydomonas the restriction sites that are highly represented are AluI, PstI, SacII, andTaqI and they serve as a sequence in the genome that are abundant and are not degenerate in the primer. They also contain a long 5′ extension called Q0 that represents a novel sequence that can be used in later rounds of PCR.

First, the genomic DNA was extracted from the culture (IBI Scientific, Mini Plant Genomic DNA kit) and checked for purity and concentration with a NanoDrop (Thermo). The first round of RESD – PCR (RESD1) was done with OneTaq DNA Polymerase (NEB) using the GC-buffer and GC-Enhance buffers. Degenerate RESD primers (see *figure 3*) were mixed with primers specific to the HYG (“down” primers: DP4 and “up” primers: UP3) or PAR (“right” primers: RB1 RB4 RB2 DPS) insert and amplified using

the Gonzalez-Ballester et al. (2005) protocol. A second round of RESD – PCR (RESD2) was accomplished by dilution of RESD1 and using similar amplification conditions except replacing the degenerate primers with the Q0 and using a primer nested within that of the primer used in the first round (e.g., DP3 or DP2 in place of DP4; *figure 2*, *figure3*). Agarose gel electrophoresis was then used to visualize DNA fragments. Further rounds of RESD – PCR with further nested primers were occasionally necessary to identify a single band. The RESD – PCR sample would be sent to be sequenced at the University of Wisconsin Biotechnology Center (Madison, WI). Once the sequences were identified, they were compared to the genome data base (Phytozome v.12.0, https://phytozome.jgi.doe.gov) to identity the gene.

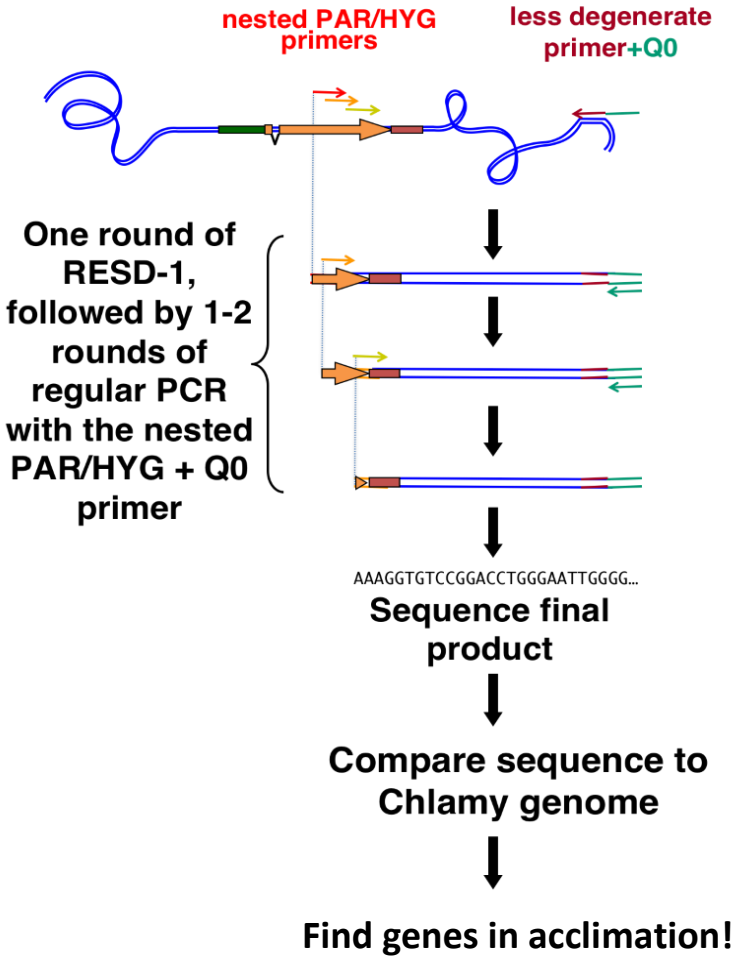

Figure 3. RESD – PCR identifies insertion sites of known DNA. Requires multiple rounds with nested primers and less degenerate primers. The small arrows represent the nested primers specific to HYG/PAR and less degenerate primer or Q0 (a 5′ extension primer). Each round of RESD amplifies the area giving a single band that can be identified. The first round uses specific nested primers and a less degenerate prime. The second round uses one of the nested primers and Q0.

Using a targeted approach to identify genes involved in limited iron acclimation Chlamydomonas mutants containing insertions in the *FOX1*, *FTR1*, *ACP1*, and *ADH1* genes were chosen to put into the screen (acquired from the Chlamydomonas Library Project, a group that is producing insertional mutants with a PAR-like DNA fragment at a large scale; Li et al., 2019). Mutant insertion sites were verified through PCR through two steps. The first verified the gene was disrupted by a DNA insertion comparing the mutant versus wild type using primers around the flanking region: the wild type will produce an expected band while the insertion mutant band will be larger by the size of PAR (or be too large to efficiently amplify). The second step verified that the appropriate PAR cassette was the cause of the insertion through the use of a cassette primer together with a flanking primer to amplify an insertion-specific sized fragment.

RESULTS

Acclimation (Recovery) of Chlamydomonas from Essential Nutrient Stresses.

The first objective of the project was to develop a functional screen that could identify acclimation to iron limitation in Chlamydomonas. The developed screen showed wildtype Chlamydomonas recovery by both a physical color change and lower absorption readings indicating a response to limited amounts of iron stress after multiple rounds of wildtype growth. Wells with iron added back returned to dark green similar to normal wildtype Chlamydomonas (*figure 4*).

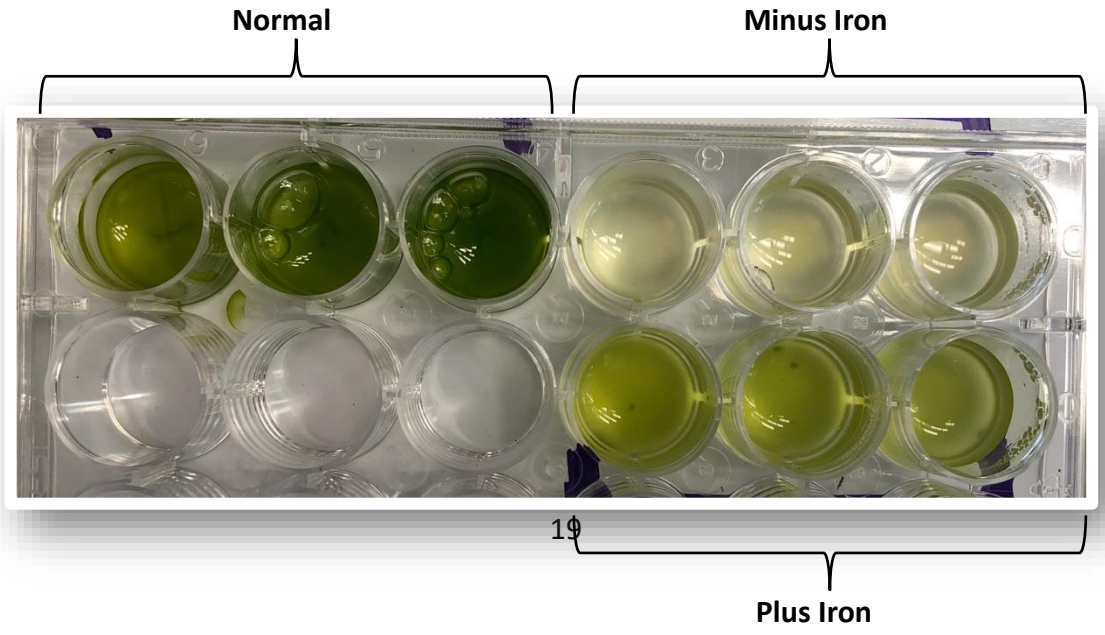

Figure 4. Chlamydomonas wildtype screen example: three replicate wells containing normal or minus iron TAP media. Three wells containing minus media that has iron added back after indication of stress (plus iron). Indication of acclimation seen by wells with iron added back by a phenotypic change (darker green).

There were many steps involved in developing a screen. First, cells were grown in normal TAP for 3-4 days in a beaker. In a 96 well plate (200µl) of normal TAP was added to 3 wells and in 6 other wells (200µl) of TAP minus iron was added. The wells were then inoculated with cells from the beaker (with a tooth pick) allowing for approximately the same amount of cells, repeated 6-8 times in one plate. It had to be established how long it takes for cells to grow under both conditions (normal and minus iron) in the wells. This gave a baseline physical color change and absorption reading to compare growth.

It was found that after 3-4 days there was a distinct color change (yellowing), indicating stress. Iron was then added back to 3 of the minus iron wells to show when/if cells recovered. Two of the rows had iron added back after 3 days of growth, 2 after 5 days and 2 after 6 days to see which showed a physical color change indicating recovery, this was repeated twice. Verification of recovery at day 3-4 was repeated approximately 10 times. It was found that 3-4 days of growth was an optimal time to add iron back for wildtype cells to recover.

To validate that the physical color change and absorption readings were because of recovery to limited iron and not more or less cells in wells, the cells were grown in small tubes with (1 mL) of normal medium. After a day of growth, the cells were counted (by a spectrophotometer absorption reading), centrifuged and resuspended in an amount of minus iron medium that allowed for the concentration of all the tubes to be the same number of cells (smallest absorption reading had 200 µl of minus iron media added). After 15 minutes in minus iron medium, the cells were added to the plate. The cells collected at the bottom of the well giving a false color and absorption reading so instead of a 96 well plate a 48 well plate (500µl of appropriate media and 25µl of cells) was used to allow for the wells to be placed on a shaker keeping the cells from settling.

Some variation was seen through the many initial screens, with unexpected variables leading to issues with the screen. Contamination with fungi and bacteria was occasionally observed, which affected both color-change and spectrophotometric measurements. Use of strict aseptic methods (not usually required due to the mainly autotrophic media used to culture Chlamydomonas that provided limited nutrients for most contaminating organisms) was instituted to help with this concern. Temperature and humidity during the growth period were also an occasional issue due to seasonal changes in the building, though this was more difficult to control due to using a light rack in the main room of the lab. Relative age of the cells at the start of the procedure was also observed to cause variation. Chlamydomonas is not well preserved in a freezer, so cultures must be maintained on TAP plates with serial passaging every 3-4 weeks. Colonies on plates after 21 days (for example) will have depleted the local nutrients of the plate more than a similar colony at 7 days and seem to show different degrees of

minor nutrient stress when added to the experiments. This variable was controlled by (individually) growing all colonies (wildtype or mutant) taken from plates in full-nutrient media for 1 day followed by washing of the cells with minus-iron media prior to beginning the experiments. Once these additional variables were better controlled, wildtype Chlamydomonas consistently indicated iron-stress by a decrease in absorption (>0.5 AU lower) in minus iron media but when iron was added back absorption increased (>0.5 AU higher) indicating recovery, iron acclimation (*figure 5*).

Overall growth and health of the cells examined through an absorption reading from the spectrophotometer for wildtype Chlamydomonas would ideally indicate acclimation by showing increase in growth (number of cells) in normal media, decrease growth in minus iron media, and decrease than increase growth in media when iron is added back (*figure 6*).

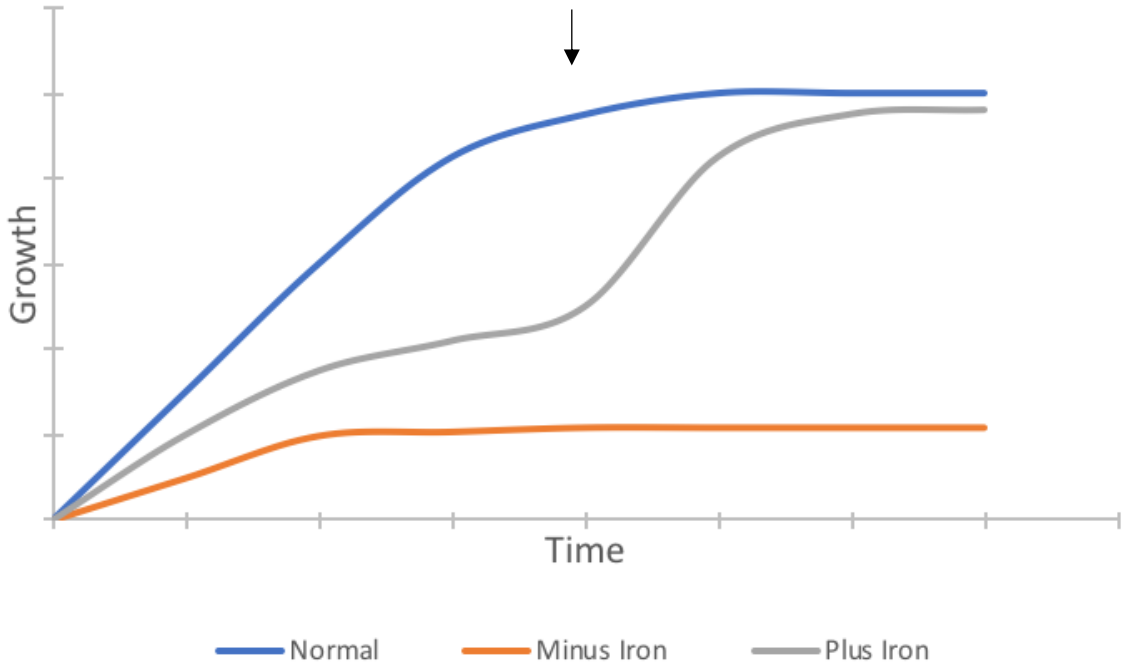

Figure 5. Ideal growth curve of developed screen testing iron acclimation. Cell growth in normal media steadily increases before leveling off due to TAP availability in each well. Cell growth in minus iron media decreases. Cell growth in plus iron (minus iron media with iron added back at indicated by the arrow) decreases and later increases indicating iron acclimation.

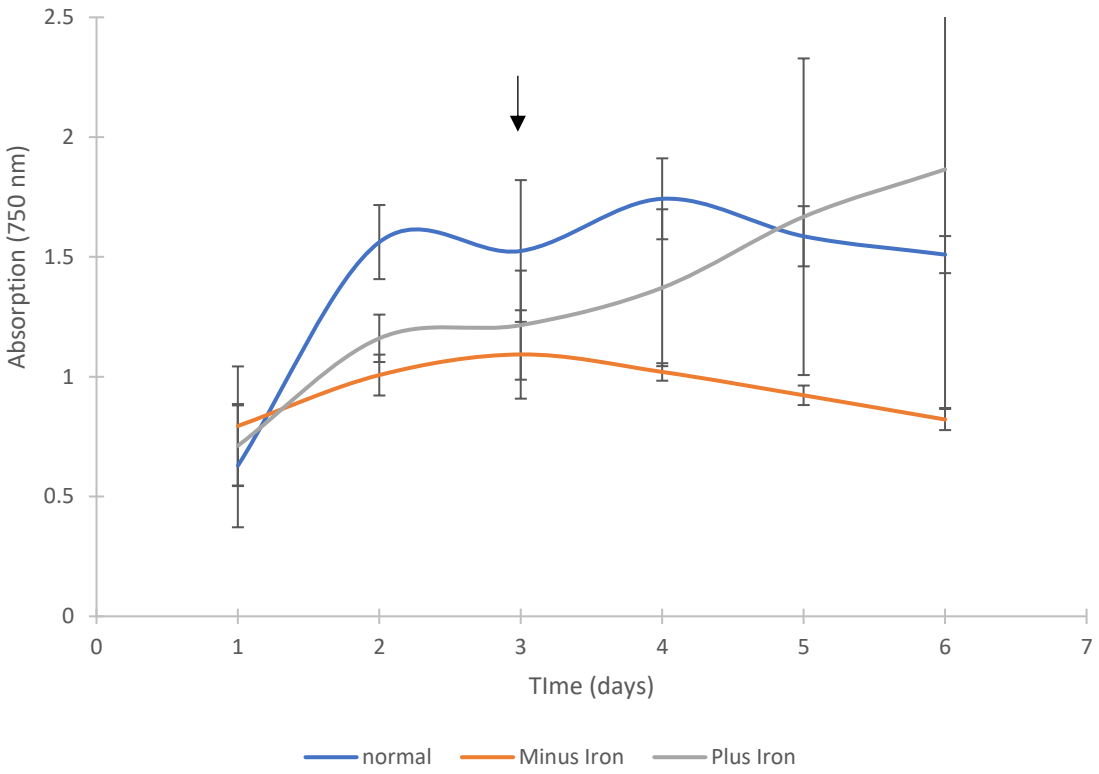

Figure 6. Growth (absorption reading) of wildtype Chlamydomonas screens over a six-day period showing recovery from iron stress indicating iron acclimation. Normal media cell growth increases while minus iron media cell growth decreases. Plus iron (minus iron media with iron added back at time indicated by the arrow) cell growth decreases then increases after iron has been added back. Error bars are standard deviation between wells (n=6).

Genetic Factors Involved in Acclimation of Chlamydomonas.

Now that a screen was possible, I began to generate mutants (non-targeted approach) to be screened by this method. I used insertional mutagenesis of DNA containing a selectable trait marker to disrupt genes creating a mutant to understand the genetic factors involved in acclimation to limited iron. Specific trait markers, HYG and PAR (antibiotic resistance) are randomly inserted into the genome through either chemical transformation or electroporation. A typical result would be ~300 colonies of potential mutants (which are mainly independent insertions). Between 5-10 colonies from each plate (approximately 20) were placed into the developed screen to test if they recover normally like wildtype Chlamydomonas. If recovery wasn't indicated by physical color change (*figure 7*) and absorption readings the mutant was amplified by RESD – PCR to identify the gene for potentially being involved in iron acclimation.

Following multiple rounds of mutagenesis and screening, six potential mutants were found showing the trait of not recovering like wildtype Chlamydomonas in two independent trails of the screen. Five mutants (named Tz, S, Az, D, and K) carrying a HYG insertion and one (B) carrying a PAR insertion were further analyzed. In the developed screen, both physical color change and absorption readings of the mutants were observed to indicate stress before addition of iron added back because stress could be indicated/seen sooner or later than wildtype depending on what gene was mutated. This meant that every new mutant's growth pattern had to be determined when to add iron back. To avoid potential false-positives, each mutant showing the lack of recovery was screened a second time to assure that the phenotype was not due to unexpected variables. Additional mutants did not repeatably show the lack of recovery over 2 trials. Use of the

electroporation method to generate more potential mutants and additional screens were halted by the COVID-19 outbreak.

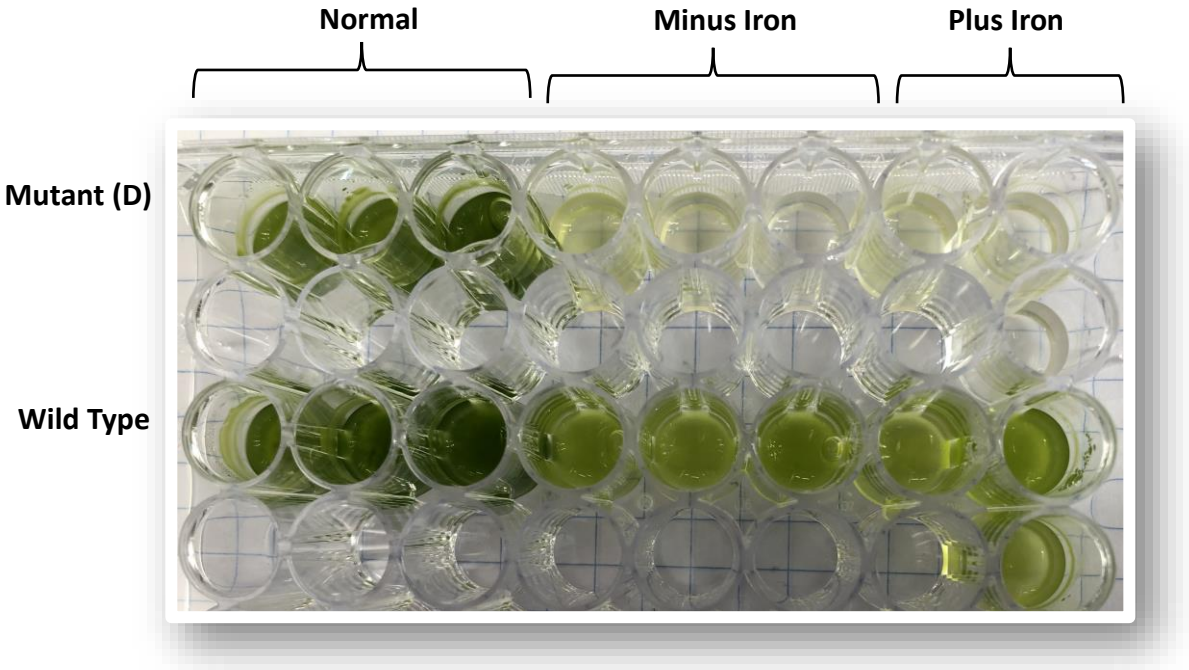

Figure 7. Developed screen with mutant (D) that isn't recovering that was further examined for potential involvement in iron acclimation. When iron was added back (plus iron) to mutant (D) the cells aren't recovering seen by yellow phenotype compared to normal media with a dark green phenotype.

The six mutants were run through RESD-PCR (*figure 8*), but multiple efforts only found smeared bands (a sign of too much template carried from the first round of RSD-PCR) or only very small bands (which reflect internal amplification of the insert that contain no genomic DNA sequence). This is not an unexpected result since RESD-PCR is subject to about a 40% success rate for mutants due the high-GC content of the Chlamydomonas genome (Gonzalez-Ballester et al., 2005; Sanderfoot lab, unpublished). General procedures are to continue to try new mutants or a second method that involves inverse PCR that has recently been shown to work by several undergraduate researchers in the lab (A. Panico, A. Gehri, G. Lentz and A.A. Sanderfoot, unpublished results). However, the COVID-19 lock-out prevented any further analysis of these mutants, so the insertion sites could not be identified.

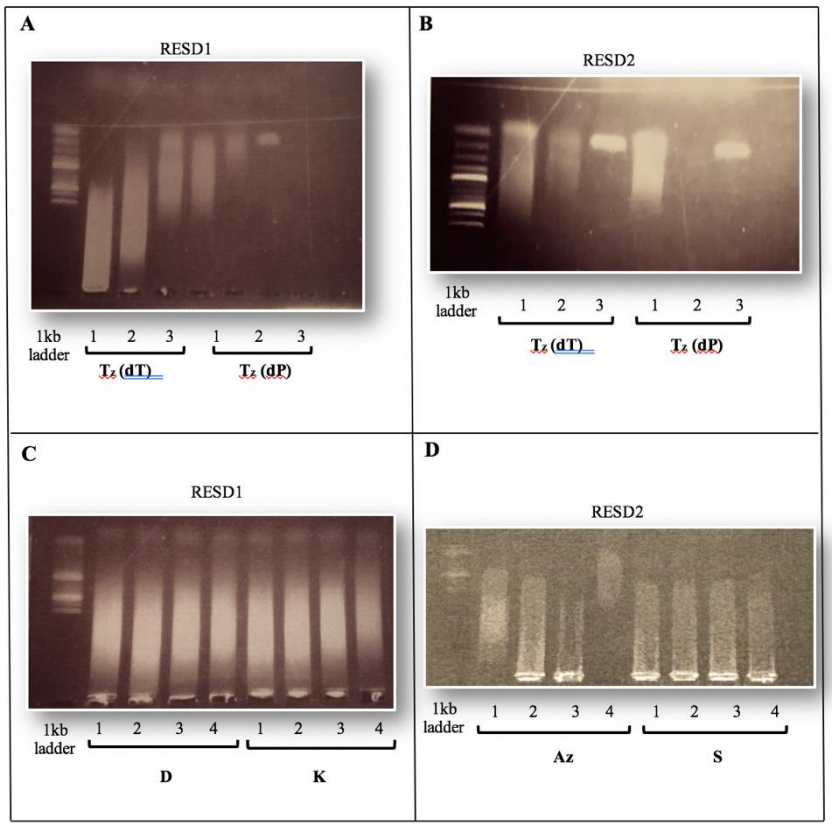

Figure 8. Representing the process of RESD-PCR. A: Genomic DNA from the mutant Tx was subjected to RESD1 with the primers DP4 and either dT or dP as indicated below the bracket. RESD1 was subsequently used for RESD2 with the primers DP2 + Q0 (1), DPS +Q0 (2), DP2 + RB3 (3). Only smears are seen except for the control lane (3, which is an internal primer set that amplifies a ~200 bp band from the HYG fragment). B: The DNA shown in the lanes of A was subsequently diluted and used for a 'RESD3' using the following primers DP3 + Q0 (1), DP2 + Q0 (2), DP2 + RB3 (3). Again, smears were seen except for ~180 bp control lane 3. C-D: Similar to panel A, genomic DNA from the D and K mutants (indicated below the bracket in C) or the Az and S mutants (panel D) were amplified with DP4 and either the dA (1), dP (2), dT (3) or dS (4) primer in RESD1 followed by DP3 +Q0 in RESD2. Only smears are seen as before.

The targeted approach of the study was to use mutants from a larger study that is creating random insertions in Chlamydomonas genes as part of a genomics project (Chlamydomonas Library Project, CLiP). This project is using a phenotype-independent method to identify many insertions throughout the genome in an attempt to provide an insertion in every gene. I conducted some research to identify some genes that I believed would have an iron-acclimated phenotype. The main metal transport uptake pathway in Chlamydomonas is fungal-like ferroxidase dependent ferric transport complex consisting of FOX1 and FTR1. FOX1 catalyzes the oxidation of Fe (II) and Fe (III). FOX1 forms a complex with FTR, a ferritin that works as the permease to transfer the ferric iron into the cytosol (Glaesener et al., 2013). ACP plays the important role of keeping iron sulfur clusters stable which are important for biosynthesis in the mitochondria (Van Vranken et al. 2016). ADH1 plays an important role in anaerobic metabolism of photosynthetic unicellular organisms. ADH1 has metal ions in the active site, iron and zinc (van Lis et al., 2017).

Mutants in the genes *FOX1*, *FTR1*, *ACP1* and *ADH1* were acquired from CLiP. However, due to the scale of the insertion process used in the CLiP method, it is essential that the insertions be verified to avoid working with the wrong mutant. For this reason, all the mutants must first be verified for the correct insertion through PCR. Verification of a mutant versus wild type was done with primers around the flanking region amplifying the region showing a larger product indicating a mutant (to be repeated with image before unable to access the lab). The second verification was done by PCR with a cassette primers for *FOX1* and *FTR1* (*figure 9*). Primers for the wildtype Chlamydomonas gene (forward and reverse primes) showing a band indicate wildtype

and if no band indicates a mutant. A wildtype forward gene primer and an up or down cassette primer result in showing a band of a certain size to verify the mutant was what was ordered (*figure 9*).

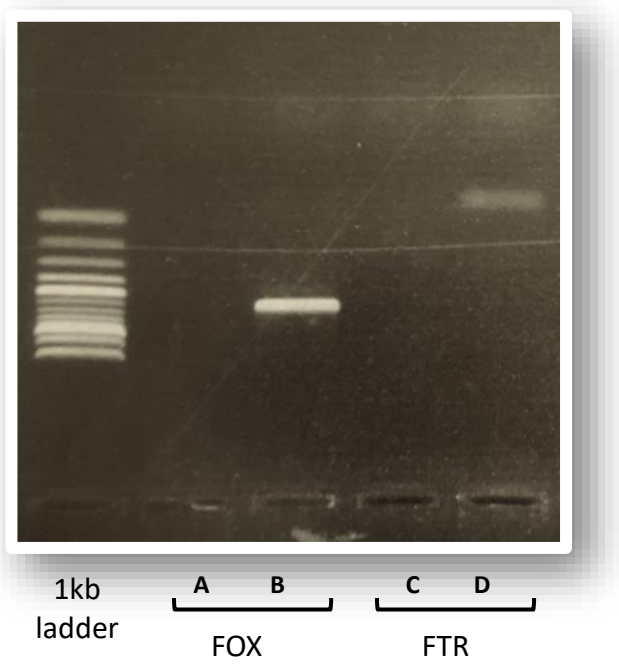

Figure 9. Gel electrophoresis image after PCR amplification check with cassette primers. A: (forward and reverse FOX primers) shows no band due to the presence of the PAR insert. B: (forward FOX primer and up FOX cassette primer) shows the expected ~ 650 bp band from the predicted insertion site. C: (forward and reverse FTR primer) shows no band due to the presence of the insert. D: (forward FTR primer and down FTR cassette primer) shows the expected 80 bp band from the predicted insertion site.

After verification of the CLiP mutants being the correct size, they would be inserted into the developed screen with wildtype Chlamydomonas to test recovery. They are expected to not show recovery since these mutants were selected with a known iron function involved. The results of this screen could also give a better baseline of recovery absorption values for comparison to other potential mutants. Due to the COVID-19 quarantine this work was prevented.

Potential for Further Larger-Scale Application of the Results.

The spring 2020 Plant Physiology course lab offered an additional opportunity to potentially expand my work outside of Chlamydomonas. During this course, tomato plant growth was analyzed when a nutrient was lacking allowing to determine which nutrients are essential. Tomato plants (3-4-week-old) were grown hydroponically allowing for better control of nutrient amounts than in soil. My specific experiment had three treatments. One plant had all the essential nutrients it needed added to survive and grow (positive control) and the other two had all essential nutrients added except for iron. Overall growth of the plants was observed. When necessary, distilled water was added to keep them from drying out. After five days, the iron lacking plants showed signs of stress (yellowing of leaves), so one of the plants lacking iron had iron added back to determine if it recovered, a similar process to the Chlamydomonas screen, leaving the other plant as a negative control. Five days after iron was added back that plant showed recovery (returning to greenness similar to the control) (*figure 10*).

Day 1

Day 5
+ Iron

Day 10

Figure 10. Tomato plants being stressed for iron and showing recovery after five days. The left jar has complete media, the middle and right jars lack added iron. Both of the jars lacking iron showed significant chlorosis after 5 days. The right jar had iron added back on the 5th day (red box) and recovered back to the green color of the control jar within 5 days.

Obviously, doing this type of screen in tomato is beyond the scope of this master's thesis, but the results show that a similar type of study could be accomplished at a larger scale in land plants. Further, as nutrient stress in crop plants affecting yield is a known factor in the field, a better understanding of the recovery will lead to a better understanding of iron acclimation.

Using a developed screen that shows Chlamydomonas stress and recovery by both physical color change and absorption reading allowed for a baseline understanding of iron acclimation. Mutants were inserted into the developed screen testing recovery by comparison to wildtype in normal media through absorbance readings and physical color changes. Several mutants didn't recover in comparison to wildtype indicating possible involvement of the disrupted gene in iron acclimation. Amplification of those disrupted genes was done by RESD – PCR so they could then be identified and sequenced to understand their function. Knowing the function of genes that are involved in iron acclimation can lead to better understanding of iron acclimation that could apply to other organisms. The developed screen could also lead to understanding other types of acclimation in Chlamydomonas and/or other organisms.

DISCUSSION and FUTURE RESEARCH

The ability to acclimate to short-term changes in the environment is essential for survival. Populations that have a better ability to acclimate to short-term changes can survive and eventually adapt through the typical mechanisms of evolution. One short-term change that can greatly impact the survival of an organisms is the varying amounts of a nutrient available to organisms during different seasons, climate change, and locations. For example, being able to acclimate to differing levels of iron specially allows for species to survive because low availability of iron in the surrounding environment is common (Vigani et al., 2016). Iron is essential for many biological processes because of its unpaired electrons which allows it to accept and donate electrons. The unpaired electrons allow iron to be involved in many functions that are essential (DNA synthesis, fatty acid metabolism, mitochondria electron transport chain, reactive oxygen detoxification, and amino acid formation) but also allows it to become toxic (Bibbin et al., 2017). Acclimation to deficiency of iron could be a part of many different pathways such as uptake, storage, distribution, and metabolism. Uptake, storage, distribution, and metabolism of iron is highly regulated by mechanisms at the transcription and posttranscriptional level (Bibbin et al., 2017, Connorton et al., 2017). Iron is also important for other organisms besides plants. Because plants are a gate way for other organisms to receive the iron they need to survive (Connorton et al., 2017), in an ecosystem it can be essential for plants to first be able to become acclimated to low iron

to allow other organism in the subsequent food web to receive the iron necessary for their survival.

The developed screen to study acclimation of Chlamydomonas to a limited amount of iron found that wildtype Chlamydomonas has the ability to recover from short term iron deficiency, i.e. to acclimate. This was shown by the phenotypic color change during growth under normal (green), minus iron (yellow), and minus iron with iron added back (back to green) conditions (*figure4, figure 6*). This phenotypic screen could be further studied to produce a quantitative analysis of recovery or acclimation. This could be done through repetition of the screen with absorption readings every day at a consistent time. This screen also indicated that there is a genetic component to the ability to acclimate by some mutants not showing recovery from iron stress. Investigation of these mutants could be key to understanding genes involved in acclimation (this step was planned to be completed but due to current world circumstances wasn't able to be). Once the mutant genes were identified for function what genetic factors allow Chlamydomonas to be acclimated to limited iron could be better understood. These genes could be analyzed for physiological and cellular function that then could be applied to further research of acclimation to other essential nutrients in Chlamydomonas. The nature of these genes could also be compared to similar genes and phenotypes in other organisms that share a common ancestor with Chlamydomonas or that have similar genes/functions.

With an understanding of genes involved in iron limitation in Chlamydomonas, it could be interesting to start varying other aspects of the environment such as pH levels, redox, forms of iron in the environment and see if and how acclimation changes. The developed screen could also be modified to focus on acclimation of other essential

nutrients, pH levels, etc. in Chlamydomonas. Another interesting focus of study could be to see what else may be taken in up in acclimation to limited iron such as other nutrients as seen in Morrissey et al., (2009). The developed screening method could be modified to use on other organisms such as bacteria and yeast to study acclimation to iron or modified for other essential nutrients. In addition, the work reported here about hydroponics in tomato plants opens up the possibility of expanding similar work to crop plants that support our food supply or other organisms in key places in food webs.

The knowledge of acclimation to iron could potentially be applied at a human level because some of the genes may lead to a further understanding of anemia. Anemia is when an organism has a deficient amount of iron. In humans iron is practically important for making hemoglobin which moves oxygen throughout the body. Long term anemia leads to severe fatigue, pregnancy complications, arrhythmia, and for more sever forms of anemia such as sickle cell anemia even death (Abbaspour et al., 2014). If some of the same or similar genes involved in iron acclimation in Chlamydomonas are also in humans, then it could lead to a better understanding of what makes humans acclimated to iron limitation to implement better treatments for anemia.

Understanding the function of the genes involved in iron acclimation of Chlamydomonas could lead to better understanding of how they contribute to stress of the environment around them. If an organism is stressed from the lack of the nutrient it is going to respond by either finding an alternative source of the nutrient within the cell or the surrounding environment and/or increase the ability to import that nutrient. By the organism responding this way it could lead to the environment being stressed and acclimating to that stress (Bijlsma & Loeschcke, 2005). For example, one organism may

deplete the availability such that other organisms may later suffer further stress affecting the diversity and balance of precarious ecosystem.

Due to current world circumstances some results expected to be completed were not able to be. These include further repetition of the wildtype in the screen along with mutants shown to not recover, a continuation of the screen to find more mutants that don't recover, and identification of the current mutants showing possible involvement in iron acclimation. Further future research would be to answer objective 3 (understand the physiological and cellular function of genes). Overall, the work of this thesis has 1) first shown that a phenotypic screen for acclimation to iron limitation was possible in Chlamydomonas, a useful system that is well studied and a model for other multicellular eukaryotes; and 2) using insertion mutagenesis, that there is a genetic basis for future work that might help with the study of the ability of all organisms to acclimatize, respond to stresses, and eventually to adapt and diversify to changing environments.

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