8-2012

# Plant-Like Cryptochrome Does Not Promote Blue Light-Induced Resetting of the Circadian Clock in *Chlamydomonas Reinhardtii*

Jonathan Howton
*Western Kentucky University*, jonathan.howton@topper.wku.edu

# PLANT-LIKE CRYPTOCHROME DOES NOT PROMOTE BLUE LIGHT-INDUCED RESETTING OF THE CIRCADIAN CLOCK IN *CHLAMYDOMONAS REINHARDTII*

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Jonathan Howton

August 2012

PLANT-LIKE CRYPTOCHROME DOES NOT PROMOTE BLUE LIGHT-INDUCED RESETTING OF THE CIRCADIAN CLOCK IN *CHLAMYDOMONAS REINHARDTII*

Date Recommended 07/27/2012

Sigrid Jacobshagen

Sigrid Jacobshagen, Director of Thesis

Claire Rinehart

Claire Rinehart

Rodney King

Rodney King

8/20/12

Dean, Graduate Studies and Research Date

# ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Sigrid Jacobshagen, for generously providing her time and experience to not only guide me through this project, but also to shape me into a better scientist. The insight and direction she has provided are invaluable. Thank you to my committee members, Dr. Claire Rinehart and Dr. Rodney King for their support. I would like to especially thank Dr. Rinehart for his assistance with both phototaxis data analysis and statistical analysis of the results.

I appreciate the support from current and former members of the Jacobshagen lab. Particularly, both lab technicians I have worked with, Jenny Stovall and Bonnie McCullagh, have spent much of their time assisting me with various aspects of my project, and they have been great people to work with. Thank you to Dr. John Andersland for providing helpful advice and suggestions, as well as always providing interesting and thought provoking questions and discussions. I greatly appreciate the guidance Dr. Shivendra Sahi provided during my TA experience; his advice has helped me become a better teacher, which is very important for my future goals.

I am grateful for the opportunities provided by Ogden College and specifically the Biology Department. I would also like to thank the Biotechnology Center for assistance and use of equipment. Additionally, thank you to the WATERS Lab for use of the dissolved oxygen electrode.

Thanks to my wife, Kristen, for being a consistently great part of my life through this challenging endeavor, and for always knowing exactly what I needed to help me through it. I also want to thank my family for a lifetime of support and encouragement, without which I would not be where I am today.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

PLANT-LIKE CRYPTOCHROME DOES NOT PROMOTE BLUE LIGHT-INDUCED RESETTING OF THE CIRCADIAN CLOCK IN *CHLAMYDOMONAS REINHARDTII*

Jonathan Howton August 2012 61 Pages

Directed by: Sigrid Jacobshagen, Claire Rinehart, and Rodney King

Department of Biology Western Kentucky University

The circadian clock is an endogenous timer that allows an organism to anticipate and properly prepare for the daily changes in the environment. This preparation occurs in the form of daily rhythms in metabolism, physiology, and behavior. These approximately 24-hour rhythms are reset upon environmental time cues such as the daily light/dark and temperature cycles. The unicellular green alga *Chlamydomonas reinhardtii* is a useful model organism for circadian clock research. It shows several well-characterized circadian rhythms of behavior, and the monitoring of its rhythm of phototaxis, or swimming towards light, has been automated. The receptors involved in entraining the clock to the daily light/dark cycle have not yet been identified in this organism. Previous research has shown that blue, green, and red light are effective in resetting the clock in *C. reinhardtii*. This study focused on identifying the blue light sensor for resetting. One possibility was reception through photosynthesis. This was tested by looking for a defect in the ability to reset the clock upon blue light in cultures treated with the photosynthesis inhibitor DCMU. It was found that photosynthesis does not mediate this process. Instead, a photoreceptor must be involved, and plant-like cryptochrome was the most probable candidate, as it is known to perform this function in higher plants. To determine if plant-like cryptochrome serves this function, available transformants of *C. reinhardtii* with an RNA interference construct designed to knockdown plant-like cryptochrome expression were used. In this study, the

transformants were screened for a reduction in cryptochrome amount using western blot analysis. The two strains with consistently the largest knockdown were tested for defects in resetting the circadian clock upon blue light pulses. Neither strain was found to be less sensitive than the parent strain to blue light induced entrainment. On the contrary, one strain was significantly more sensitive than the parent strain, which suggests a possible inhibitory role for plant-like cryptochrome in the photoentrainment of the clock to blue light in this organism.

# CHAPTER I

## Introduction

The circadian clock, which is found in practically all eukaryotes and some prokaryotes, is a biochemically based endogenous timekeeper that allows an organism to sense the passage of time. The clock synchronizes many metabolic, physiological, and behavioral processes with the daily rhythms of light and temperature; and these processes retain their approximately 24-hour rhythms even in the absence of environmental time cues (Werner 2002). For example, plants assemble their photosynthesis apparatus just before dawn to make optimal use of available light energy (Tiaz and Zeiger 2006). This self-sustaining rhythmicity allows an organism to anticipate daily changes so that it may both overcome and take advantage of the periodic nature of the environment (Pittendrigh 1993).

Conceptually, the circadian clock consists of three major components: the central oscillator, the input pathway, and the output pathway. The central oscillator acts as the pacemaker of the clock by producing a free-running oscillation with a period of approximately 24 hours. The output pathway coordinates clock-regulated processes with the timing of the central oscillator. Since the purpose of the clock is to allow an organism to anticipate regular daily environmental changes, it is necessary for the clock to remain in synchrony with daily environmental oscillations. The input pathway preserves this synchrony through its ability to entrain the clock based upon environmental time cues, such as the daily light and temperature oscillations. In reality, this concept of a three-component system is an over simplification of the clock, as the components are able to

feed back on each other; this provides a flow of information that is not simply unidirectional (Devlin 2002).

*Chlamydomonas reinhardtii* is a unicellular green alga that is widely used as a model organism in many areas of research. Its well-characterized, single, cup-shaped chloroplast serves as an excellent model for chloroplast assembly and physiology due to the organism's ability to grow heterotrophically, as this allows for experiments that may disrupt photosynthesis (Rochaix 2001). This would not be possible in obligate autotrophs, such as higher plants, as they would have no means of obtaining energy. *C. reinhardtii* also serves as a model for cilia/flagella assembly and physiology due to its two well-characterized flagella. The study of its flagella has led to a better understanding of cilia-related human diseases (Silflow and Lefebvre 2001). Additionally, this alga has been used as a model for studying metabolism and nutrient acquisition (Harris 2001).

*C. reinhardtii* is a useful model organism for circadian clock research as well. At the time when circadian rhythm mutants were first isolated in *Drosophila* and *Neurospora* (Konopka and Benzer 1971; Feldman and Hoyle 1973), altered period mutants were also isolated in *C. reinhardtii* (Bruce 1972). Bruce was able to measure the period of the clock by measuring the circadian rhythm of phototaxis, or swimming toward light, which he had demonstrated previously (Bruce 1970). This rhythm was later shown to be a true endogenous rhythm, as it was maintained without any time cues in the completely constant condition of outer space (Mergenhagen and Mergenhagen 1987). This rhythm is also particularly useful because it can be measured in an automated fashion, and, recently, the automated measurement and analysis of this rhythm have been optimized (Gaskill et al. 2010). In addition to phototaxis, circadian rhythms of

chemotaxis, cell division, and some gene expression occur (Sjoblad and Frederikse 1981; Goto and Johnson 1995; Kucho et al. 2005).

All clocks in all systems are known to respond to light in a time-of-day specific manner; the time at which the light is perceived determines the extent and direction of clock resetting (Devlin and Kay 2001). If an organism is treated with a light pulse during its subjective night, it will adjust its free running clock to correct the apparent discrepancy. A pulse given in the first half of the subjective night will result in a phase delay because it appears as if the timing of the clock is ahead of the environment; if the pulse is given in the second half of the subjective night, it appears as if the timing of the clock is behind the environment, so a phase advance occurs (Johnson et al. 2003). Additionally, the clock only resets upon certain wavelengths of light due to the particular absorption characteristics of the photoreceptor proteins that mediate the response. For instance, the *Drosophila* clock is entrained by blue light (Frank and Zimmerman 1969), while the *Arabidopsis* clock is entrained by both red and blue light (Millar et al. 1995).

Currently, the molecules involved in perceiving light as a time cue are unknown in *C. reinhardtii*. In other organisms this function is performed by photoreceptors, which are proteins that perceive light and transduce a subsequent signal so that the cell can respond to changes in light (Somers et al. 1998; Hattar et al. 2002). This function is performed by a single cryptochrome in *Drosophila* (Emery et al. 1998; Stanewsky et al. 1998), and melanopsin in mammals (Hattar et al. 2002). In *Arabidopsis*, red light-induced entrainment occurs through phytochromes A and B, and blue light-induced entrainment occurs through phytochrome A and cryptochromes 1 and 2 (Somers et al. 1998). While the particular photoreceptor may vary between organisms, it is consistent

that a photoreceptor is used to detect light as a time cue. Therefore, it is likely this is true in *C. reinhardtii* as well.

Since photoreceptors absorb specifically at certain wavelengths, determining which wavelengths cause the clock to reset can narrow down potential photoreceptors. In order to determine the wavelengths of light that reset the clock in *C. reinhardtii*, action spectra for resetting the clock were measured. An action spectrum for resetting the clock in dark-adapted CW15, a cell wall deficient strain, showed that both 520-540 nm green light and 660 nm red light induced a resetting of the clock (Kondo et al. 1991). Surprisingly, there was very little response to blue light, which, as mentioned previously, is effective in both *Arabidopsis* (Millar et al. 1995) and *Drosophila* (Frank and Zimmerman 1969). A recently determined action spectrum (Figure 1) using wild-type cells confirmed the resetting response to these two wavelength regions, but also demonstrated that 440nm blue light is highly effective in resetting the clock as well (Forbes-Stovall 2011). Photosynthesis was shown to be involved in resetting the clock in *C. reinhardtii* CW15 cultures kept under constant dim light (Johnson et al. 1991). It also shows peaks in its action spectrum from 450 nm to 480 nm in the blue range, and from 650 nm to 670 nm in the red range (Tiaz and Zeiger 2006). Consequently, photosynthesis may perceive these wavelengths as time cues for resetting the clock in *C reinhardtii*, though it usually requires a light intensity with a higher fluence rate to induce a photosynthesis response than used in the latter action spectrum. For example, the study in which light-adapted CW15 cultures showed photosynthesis was involved in photoentrainment used fluence rates of 50-60 μmol $m^{-2}$ $s^{-1}$ (Johnson et al. 1991), but the recent action spectrum only used a fluence rate of 0.082 μmol $m^{-2}$ $s^{-1}$ or less (Forbes-

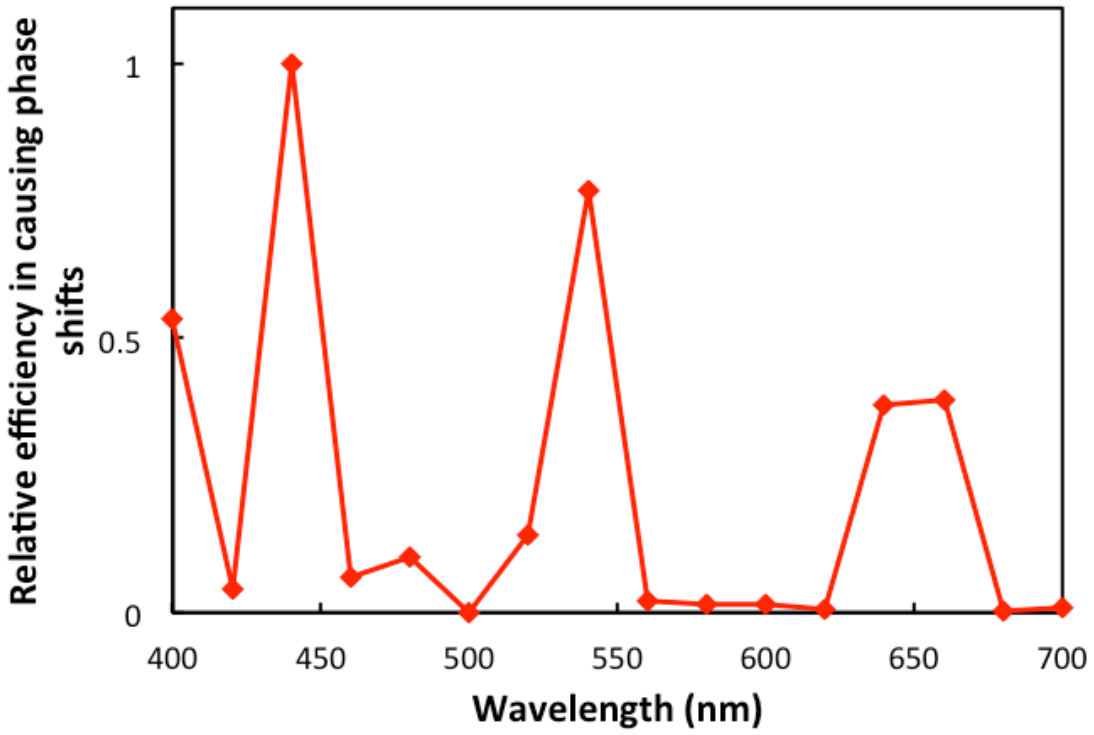



**Figure 1. Action spectrum for photoentrainment of wild-type *C. reinhardtii*.** The reciprocal of the light intensity that caused a phase delay of 2 CT units was plotted against the wavelength. A CT unit represents 1/24 of the measured circadian period. The reciprocal of light intensity was normalized against the maximal response at 440nm, which was set at 1 (Forbes-Stovall 2011).

Stovall 2011). Because of this, it is more likely that a photoreceptor mediates this response in *C. reinhardtii*.

There are several photoreceptors that have been characterized in *C. reinhardtii*. There is a single phototropin (Huang et al. 2002), a plant-like cryptochrome (Small et al. 1995), an animal-like cryptochrome (Mittag et al. 2005), and several rhodopsins (Deininger et al. 1995; Sineshchekov et al. 2002; Kateriya et al. 2004). Phototropin and both cryptochromes absorb blue light, while the rhodopsins absorb blue-green light. A strong candidate for the photoreceptor that mediates blue light-induced entrainment of the clock in *C. reinhardtii* is the plant-like cryptochrome because its amino acid sequence is very similar to *Arabidopsis* cryptochromes (Small et al. 1995), which are known to perform this function (Somers et al. 1998). Additionally, the recently characterized animal-like cryptochrome is a very strong candidate for the photoreceptor that performs this function because it detects both blue and red light, and even regulates several core clock genes upon doing so (Beel et al. 2012). A homolog to this gene has been found in a diatom (Coesel et al. 2009) and the green alga *Ostreococcus* (Heijde et al. 2010), which have been shown to have both cryptochrome photoreceptor activity and photolyase activity. However, at the beginning of this study, little was known about this photoreceptor and it was actually annotated as a 6-4 DNA photolyase in the genome, so only the plant-like cryptochrome was considered.

Cryptochromes are blue light and UV-A photoreceptors that share sequence similarity to DNA photolyases, but lack the DNA repair mechanism of the photolyase; additionally, cryptochromes have a C-terminal extension that is not found in photolyases (Cashmore 2003). The first cryptochrome described for *C. reinhardtii* was identified due

to its ~43% identity to well-characterized photolyases and ~49% identity with *Arabidopsis* cryptochrome (Small et al. 1995). Currently, there is no known function for this plant-like cryptochrome in *C. reinhardtii*, but it has been determined to function in the circadian clocks of other organisms. For example, it has been implicated in the input pathway of *Arabidopsis* (Somers et al. 1998). Both *Arabidopsis* cryptochromes function by modulating the activity of the ubiquitin ligase COP1 in a light dependent manner; the C-terminal CCT domain of each *Arabidopsis* cryptochrome binds COP1 constitutively, and the ubiquitin ligase activity of COP1 is blocked by light activation of cryptochrome (Wang et al. 2001). In contrast, mammalian cryptochrome plays a non-photoreceptive role as a component of the central oscillator (Kume et al. 1999), but also acts as a photoreceptor involved in the input pathway (van der Horst et al. 1999). *Drosophila* also uses cryptochrome within both the central oscillator (Krishnan et al. 2001) and input pathway (Emery et al. 1998). Since cryptochrome is a blue light photoreceptor and is involved in photoentrainment of other organisms, it is a strong candidate for this photoreceptor function in *C. reinhardtii*.

This study focuses on determining if plant-like cryptochrome is the photoreceptor that mediates the blue light-induced resetting of the circadian clock in *C. reinhardtii* because it is a blue light photoreceptor that has been found to perform this function in closely related higher plants (Somers et al. 1998). Although it was unlikely for photosynthesis to be involved, it was tested to confirm this. To do so, cultures were treated with the photosynthesis inhibitor DCMU. DCMU blocks photosynthesis by binding to the plastoquinone binding site of photosystem II, which halts photosynthetic electron transport (Taiz and Zeiger 2006). Therefore, it will block any photosynthesis-

mediated responses. Once it was shown that photosynthesis was not involved, it was even more likely that a photoreceptor served this role. To test if cryptochrome was involved, a previous student designed an RNAi construct to knockdown cryptochrome expression. This student also transformed the construct into *C. reinhardtii* and isolated several transformants. In this study, the transformants were screened for reduced cryptochrome expression, and two transformants with consistently lower cryptochrome expression were tested for defects in their ability to reset the clock in upon blue light.

# CHAPTER II

## Materials and Methods

### *Chlamydomonas* strains and growth conditions

This study utilized *Chlamydomonas reinhardtii* strain CC124, which was obtained from Christoph Beck (Albert-Lubwig University, Freiburg, Germany); and strain CC48, which was obtained from the Chlamydomonas Center core culture collection at http://www.chlamy.org/strains.html. *C. reinhardtii* axenic stock cultures were maintained on slants comprised of YA agar medium (Harris, 1989). Working cultures were inoculated from the slants either into liquid 0.3 HSM medium and grown autotrophically, or into liquid TAP medium and grown mixotrophically (Harris, 1989). Cultures were grown on an orbital shaker at 150 rpm in 125 ml Erlenmeyer flasks with 50 ml of medium. The conditions in the room were maintained at 20˚C with a 12-hour light/12-hour dark cycle.

Light pulse experiments used cultures in 1L bottles of 0.3 HSM that were inoculated from the working cultures at 1 X $10^4$ cells/ml. These were grown in an incubator at 20˚C, aerated by bubbling with an aquarium pump, and illuminated for at least 4 cycles with a 12-hour light/12-hour dark cycle opposite to the naturally occurring one. GE plant and aquarium wide spectrum fluorescent bulbs provided light inside the incubators with 85 µmol $m^{-2}$ $s^{-1}$ from each side in incubator #1 and 70 µmol $m^{-2}$ $s^{-1}$ from each side in incubator #2. All cultures of CC48 also contained L-arginine at a concentration of 0.2 mg/ml. Cell concentrations were determined using a hemacytometer (Harris, 1989).

In experiments involving DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, cat.# D2425, Sigma-Aldrich), cultures were treated with a 1:1000 dilution of either ethanol or 6mM DCMU in ethanol at the start of the dark phase immediately before being placed in a dark box. Immediately after light pulse treatment, cultures were centrifuged at maximum speed in a clinical centrifuge for 2 minutes and resuspended in conditioned medium from a replicate culture. The entire duration that cultures were exposed to DCMU was therefore 5 h and 15 min.

To freeze a culture aliquot for storage, cells were grown in 50 ml TAP in a 125 ml Erlenmeyer flask on an orbital shaker to a concentration of about 1 X $10^6$ cells/ml. A volume containing $10^7$ cells was centrifuged in a 15 ml corning tube at medium speed (setting 4) in a clinical centrifuge (Damon/IEC) for 5 minutes. Under sterile conditions, the cells were resuspended in 1 ml of fresh TAP medium. In a 1.8 ml cryotube, 250 µl of the 1 ml culture was combined with an equal volume of fresh TAP medium containing 6% (v/v) methanol. The cryotubes were placed into a freezing container (5100 Cryo 1˚C Freezing Container "Mr. Frosty", NALGENE), which was filled with isopropanol, and the container was placed in a -80˚C freezer for 69 minutes. Tubes were then transferred to liquid nitrogen for storage.

In order to thaw frozen cultures again, cryotubes were removed from liquid nitrogen and immediately placed into a 35˚C water bath for two minutes with gentle shaking. The thawed cultures were then added to 10 ml of TAP in a culture tube and placed under constant light for 17 hours with gentle shaking (Labquake Shaker and Rotisserie, Barnstead/Thermolyne). Afterwards, the culture was split so that 6 ml was

used to inoculate a TAP plate and the remaining 4 ml was used to inoculate liquid 0.3 HSM and TAP stock cultures.

Measuring oxygen evolution

Cultures were grown in 1 liter bottles of 0.3 HSM autotrophic medium in a 20˚C incubator with a 12-hour light/12-hour dark cycle to a concentration of approximately 2 X $10^6$ cells/ml. A culture aliquot of 300 ml was then transferred to a HACH disposable 300 ml BOD bottle and either 300µL of ethanol or 300µL of 6 mM DCMU in ethanol added so that the final concentration of DCMU was 6 µM. The bottle was capped with a glass stopper and placed on an orbital shaker for 1 minute to mix the culture well. Dark controls were made by wrapping bottles of ethanol treated cultures with aluminum foil. Dissolved oxygen was then measured using an oxygen electrode (HQ40d multi, Hach). Samples were capped and placed on an orbital shaker between readings. The rate of change in oxygen concentration was determined by taking the slope of the plotted oxygen concentration vs. time curve. These rates were normalized for the varying culture concentrations by dividing the rate by the cell concentration of the culture used. To determine the rate of oxygen evolution, the average rate of change in oxygen concentration from the dark controls was subtracted from each average rate of change in oxygen concentration from the ethanol and DCMU treated cultures.

Light pulse applications

The light pulse device (Figure 2) used a 150W solar simulator (Spectra-Physics, Stratford) to administer light to cultures. This light beam first interacted with a dichroic

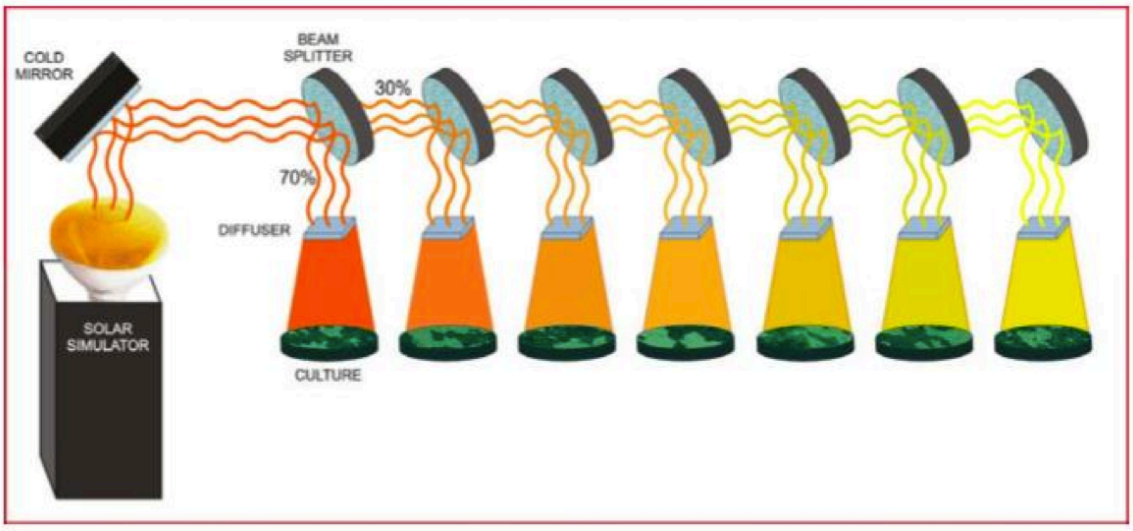



**Figure 2. Light Pulse Device.** The beam of light produced by the solar simulator is directed to a cold mirror that only reflects the visible light to a series of beam splitters, while UV and IR wavelengths pass through. Between the cold mirror and beam splitters, bandpass filters and neutral density filters can be inserted to restrict the wavelength and intensity, respectively, of the light beam. Each beam splitter reflects 70% of the light beam down to the culture through a 30˚ diffuser, while the remaining 30% is transmitted to the next beam splitter. (Figure from Gaskill, 2008).

cold mirror so that UV and IR wavelengths passed through, and only visible light (400nm-700nm) was reflected to the cultures. A 440nm narrow bandpass interference filter with a full bandpass width of 10 nm at half-maximum transmittance (Newport, Irvine) was inserted immediately after the cold mirror to allow only this wavelength to pass through. Neutral density filters were inserted as well to reduce the light intensity of the beam interacting with the cultures. If not specified otherwise, a 2.0 neutral density filter was used with cultures of CC124, and a 1.0 neutral density filter for all other strains. Next, the light beam passed through a series of seven beam splitters, each of which were designed to reflect 70% of the light beam onto the culture, while transmitting 30% of the beam to the next beam splitter. Just before reaching the culture, the light beam traveled through a 30˚ diffuser to ensure the light was distributed evenly over the culture.

Cultures were grown in an incubator as described above until reaching late log phase concentrations between 1.5 X $10^6$ and 2 X $10^6$ cells/ml. At the end of the last light phase, 3 ml culture aliquots were placed into 35 mm petri dishes (Corning, catalog # 08-772-30) under sterile conditions. Each plate was then wrapped with parafilm to prevent liquid from spilling during manipulation. At the start of the dark phase, the plates were arranged in stacks of seven, which were wrapped in foil, placed in a cup, and covered with an x-ray film pouch to prevent exposure to light; these were immediately placed into a dark box.

Thirty minutes before a light pulse, the solar simulator on the light pulse device was turned on so that it could warm-up. Ten minutes before the pulse, the solar simulator was turned off and, under complete darkness, seven aliquots of culture were placed into the appropriate slots of the light pulse device. The solar simulator was then turned back

on. At the time of light pulse, the shutter was lifted, which allowed the beam to illuminate the cultures. At the end of the pulse, the shutter was closed and the solar simulator was turned off. The cultures were then removed from the device and placed back into a stack of seven, wrapped in foil, placed back into the cup, covered with the x-ray film pouch, and placed back into the dark box. Control cultures were also kept under these conditions, but they remained in the dark box throughout the light pulse. Cultures were placed into the phototaxis machine 23 hours after initial placement in the dark box. All manipulations of cultures after initial placement in the dark box were performed in complete darkness. Light pulse intensities were previously determined (Forbes-Stovall 2011).

Phototaxis data collection

Monitoring of the circadian phototaxis rhythms was performed as previously described (Gaskill et al. 2010). Briefly, the automated machine delivered a beam to the center of the culture from a blue green LED (507nm maximum) for 15 minutes to elicit phototaxis in the culture. This was repeated every hour for the duration of the phototaxis data collection time, which was at least 96 hours. A photosensor on the opposite side of the culture detected the amount of light that passed through. The positive phototaxis induced by the light beam caused the cells to accumulate in the light beam, which in turn reduced the amount of light reaching the sensor. Consequently, the transmittance of the light beam is inversely proportional to the amount of phototaxis occurring at a particular time point. When the transmittance is plotted over time, a sinusoidal graph is produced in which the points of lowest transmittance represent the times of highest phototaxis. The

algorithm used to analyze the rhythm with respect to period and phase has been described (Gaskill et al. 2010).

Definition of units used in circadian clock research

The time an event occurs during an organism's regular 12-hour light/12-hour dark cycle is often expressed in "LD" units, where LD0 is the beginning of the light phase and LD12 is the beginning of the dark phase; and each LD unit represents an hour. Alternatively, when an organism is in a free-running state without a time cue, units of time are often expressed in circadian time units, or CT units. Since circadian rhythms are not exact 24-hour rhythms, CT units are not expressed in absolute hours. Instead, the period of the rhythm is divided into 24 equal CT units. Therefore, for an organism in free-running conditions, the beginning of subjective day is expressed as CT0, and the beginning of subjective night is expressed as CT12.

Culture collection for protein extraction

Plant-like cryptochrome is degraded in the light with a reported half-life of 15 to 20 min (Reisdorph and Small 2004), so protein samples had to be harvested in complete darkness. To allow for collection in the dark, 50 ml of culture in a 125 ml Erlenmeyer flask was wrapped in aluminum foil and placed on an orbital shaker at the beginning of the 12-hour dark phase. Samples were collected either 15 hours after the beginning of the dark phase or at the time of the light pulse. Cultures harvested 15 hours after the beginning of the dark phase were grown in TAP medium to a concentration between 4 X $10^6$ and 6 X $10^6$ cells/ml before being directly wrapped in aluminium foil. Cultures

harvested at the time of the light pulse represented a 50 ml aliquot of the 1 L incubator culture used for a light pulse experiment, with the aliquot transferred to the 125 ml Erlenmeyer flask just before being wrapped in aluminum foil. During the collection in complete darkness, 10 ml of culture was transferred to a 15 ml Corning tube, spun in a clinical centrifuge at maximum speed for 90 seconds, and the supernatant poured off. Only for the samples collected at the time of the light pulse, an additional 10 ml of culture was added, and those tubes were then spun again at maximum speed for 90 seconds; the supernatant was discarded. Four hundred µl of Laemmli final sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 50 mM dithiothreitol, 10% glycerol, 0.001% bromophenolblue) was added to each tube, and the cells were resuspended by vortexing at maximum speed for 30 seconds. The sample was then flash frozen in liquid nitrogen, the room lights turned on, and the sample placed in a -80˚C freezer for storage.

Protein sample preparation

Samples were removed from -80˚C freezer and immediately vortexed upon thawing. Immediately after vortexing, the sample tube was placed in ice water and treated with a sonifier (vibra-cell VCX130, Sonics and Materials, Inc.) with amplitude set at 40%. Five 10-second pulses were given per sample, with a 20 second cooling period between pulses. Sonicated samples were transferred to 1.5 ml microfuge tubes and placed in boiling water for 1 minute. Samples were then placed on ice for two minutes, and centrifuged at 17,000 x g for 2 minutes. The supernatant was transferred to a fresh 1.5 ml microfuge tube, while the pellet was discarded. Protein concentration was determined using the formula: Total Protein concentration (mg/ml) = (1.55 *$A_{280}$) -

($0.76*A_{260}$). Absorbance was measured using a UV-mini 1240 spectrophotometer (Shimadzu). Protein samples were stored at -80˚C.

Western blot analysis

SDS polyacrylamide gels (Resolving gel: 7.5% acrylamide/bisacrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.5 mg/ml ammonium persulfate, 0.03% TEMED; Stacking gel: 3.9% acrylamide/bisacrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.6 mg/ml ammonium persulfate, 0.1% TEMED) were cast using a Mini-PROTEAN 3 casting module (Bio-Rad). Gels were placed into a Mini-PROTEAN 3 electrophoresis module (Bio-Rad). The module was filled with 350 ml electrophoresis buffer (24.8 mM Tris, 134.2 mM glycine, 0.1% SDS). The gels were loaded so that the same amount of protein was placed into each lane with no volume exceeding 15µl. The total protein loaded per lane in each gel ranged from approximately 60 to 90 µg. Gels were run at 160V for 50 minutes. Gels and Hybond-C membranes (Amersham Biosciences) were incubated in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol) for 15 minutes before the transfer apparatus (Mini Trans-Blot Cell, Bio-Rad) was assembled according to the manufacturer's instruction. Protein was transferred at either 100V for 1 hour at room temperature or 30V overnight in a 4˚C cold room. Gels were stained using Coomassie Brillant Blue G-250 (Sigma-Aldrich) as described (Neuhoff et al. 1988). Membranes were stained for total protein with Ponceau S solution (0.2% Ponceau S (Fisher Scientific), and 1% acetic acid) for 5 minutes, and then destained briefly with water until background stain was removed. Ponceau stain was completely removed prior to incubation in blocking solution using water and PBS-T (1 X PBS, 0.1% Tween-20). Membranes were blocked for 1 hour using PBS-TM (3% dried, nonfat milk in PBS-T).

Membranes were then incubated for 1 hour with primary antibody diluted in PBS-TM. A dilution of 1:5,000 was used for the anti-cryptochrome antibody, which was obtained from Gary Small (University of South Dakota, Vermillion, South Dakota). A dilution of 1:750,000 was used for the control antibody against the beta-subunit of the chloroplastic ATP synthase, which was obtained from Francis-André Wollman (Université Pierre et Marie Curie, Paris, France). Membranes were then rinsed twice, washed once for 15 minutes, and washed twice for 5 minutes with PBS-T. Membranes were then incubated for either one-hour at room temperature or overnight at 4˚C in secondary antibody (Anti-Rabbit IgG (whole-molecule)-Peroxidase developed in Goat, Sigma-Aldrich), which was a 1:5000 dilution in PBS-TM. Membranes were then rinsed twice, washed once for 15 minutes, and washed twice for 5 minutes with PBS-T. A piece of filter paper was placed onto a sheet of plastic wrap and generously coated with a chemiluminescence solution (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, 0.2 mM p-coumaric acid, 0.0000915% hydrogen peroxide). Air bubbles were rolled out using a plastic pipette. The membrane was then placed on the filter paper and wrapped with the plastic wrap so that only a single layer covered the membrane. Air bubbles were gently pushed out using a kimwipe. Chemiluminescence of the membranes was detected using a digital imaging system (FluorChem HD2, Alpha Innotech). Quantitative analysis was performed using the "band analysis" tool within the FluorChem HD2 software. Using this tool, boxes of equal size were placed around bands of interest as well as an area without a band to serve as a background. The background spot density was subtracted from the band of interest so that only the spot density produced by the band of interest was considered. Protein amounts were then determined relative to parent strain levels. These parent strain levels

were based upon a single band per membrane in Figures 7 and 11, and upon the average of two independent bands in Figure 13.

Culture collection for RNA extraction

At the start of the dark phase, a 50 ml aliquot of the culture used for a light pulse experiment was placed into a 125 ml Erlenmeyer flask, wrapped in aluminum foil, and placed on an orbital shaker. At the time of the light pulse, the 50 ml aliquot of culture was added to a 250 ml centrifuge bottle containing 20 g of ice in complete darkness. The bottle remained on ice for the duration of the light pulse before being removed from darkness and centrifuged at 4,600 x g at 4˚C for 5 minutes. The supernatant was discarded and the pellet was resuspended in the remaining liquid. The culture was then transferred to a 50 ml Corning tube and centrifuged at 970 x g at 4˚C for 10 minutes. The supernatant was discarded and the sample was frozen in liquid nitrogen. The sample was stored at -80˚C.

Total RNA extraction

Seven ml of Trizol reagent (cat.# 15596-026, Ambion) was added to each pellet and the cells were lysed by pipetting the solution up and down. The samples were incubated at room temperature for 5 minutes. Chloroform was added to each sample in the amount of 1.4 ml, mixed by shaking for 15 seconds, and incubated at room temperature for 3 minutes before being centrifuged at 12,000 x g for 15 minutes at 4˚C in glass Corex tubes. Approximately 3.5 ml of the aqueous layer was removed from each tube and combined with 3.5 ml isopropanol in a fresh Corex tube. The solutions were

mixed by inverting and incubated at room temperature for 10 minutes before being centrifuged at just below 12,000 x g for 10 minutes at 4˚C. The supernatant was discarded and 7 ml 75% ethanol was added to each pellet and rinsed by vortexing. Samples were centrifuged for 5 minutes at 4˚C and the supernatant was discarded. The pellet was air dried for 10 minutes, and then 400 µl DEPC-treated water was added and mixed with the pellet by pipetting. To dissolve the pellet completely, samples were incubated at 60˚C for 10 minutes, and then transferred to 1.5 ml microfuge tubes. Total RNA was quantified using a NanoDrop. RNA was converted to cDNA using a high-capacity RNA to cDNA conversion kit (Part No. 4387406, Applied Biosystems) with random primers, and the manufacturer protocol was followed. Remaining RNA samples were stored at -80˚C.

Quantitative real-time PCR (qRT-PCR)

Primers were designed for the CPH1 gene of *C. reinhardtii* using the Roche Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Primers were designed so that they overlapped an exon-exon boundary. Two CPH1 primer pairs were ordered from Integrated DNA Technologies. One spanned the exon 1/exon 2 boundary (Forward: TTAAGACAGCCGTTGTGTGG, Reverse: ATGAACACGGGTATCCACATT), and the other spanned the exon 3/exon 4 boundary (Forward: GGTGGACTGGTTCTTCACG, Reverse: GTGCTCCAGCTCGCTGAT). Control primers used were for 18S rRNA #8 (Forward: GGGAGCATGAGAGATGGCTA, Reverse: TCGGGATTGGGTAATTTGC), 18S rRNA #77 (Forward: GATTGAGAGCTCTTTCTTGATTCTG, Reverse:

AGGCAACCCACCAACTAAGA), and CBLP (Forward: TCGCTGTGGGACCTGGCTGA, Reverse: GCCTTCTTGCTGGTGATGTTG).

qRT-PCR reactions were carried out in a 96-well optical reaction plate with barcode (Part No. 4306737, Applied Biosystems). The 15 µl reaction mixtures consisted of 600 µM forward primer, 600 µM reverse primer, 1X Power SYBR Green PCR Master Mix (Part No. 4367659, Applied Biosystems), and 0.56 ng/µl cDNA. A real time PCR system (7300 Real Time PCR System, Applied Biosystems) was used to run both an amplification reaction and a dissociation curve. The amplification reaction consisted of 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 95˚C and 1 minute at 60˚C. Dissociation curves indicated a single amplicon for all primers.

Statistical analysis

Statistical analysis of phase shift curves was performed by an ANOVA through the Mathematica program.

# CHAPTER III

## Results

### Measuring effectiveness of photosynthesis inhibitor

One possible way for *C. reinhardtii* to detect blue light as a time cue for shifting its circadian rhythms is through photosynthesis. To exclude this possibility in favor of a photoreceptor-mediated process, the inhibitor DCMU was used to block photosynthetic electron transport during a light pulse. A concentration of 6 μM DCMU in the culture medium was previously found to block 95% of photosynthetic activity in a mutant strain of *C. reinhardtii* (Kondo et al. 1991). In order to determine whether it was effective in strain CC124, the dissolved oxygen concentration was measured over time as an indicator of photosynthetic activity for cells kept under light conditions and treated with either ethanol as a control (Figure 3) or DCMU in ethanol (Figure 4). To determine oxygen consumption due to aerobic respiration, oxygen concentration was also measured for cells treated with ethanol and kept in the dark (Figure 5). Based on these measurements, it was determined that the 6 μM DCMU treatment blocked 66% of photosynthesis activity compared to the control.

### Photosynthesis does not affect resetting

To determine whether photosynthesis is mediating the blue light dependent resetting of the clock, 440 nm blue light pulses were given at LD19 to cultures with and without DCMU treatment, and their ability to reset their circadian rhythm of phototaxis was measured. LD19 was previously shown to be the optimal time for this strain to

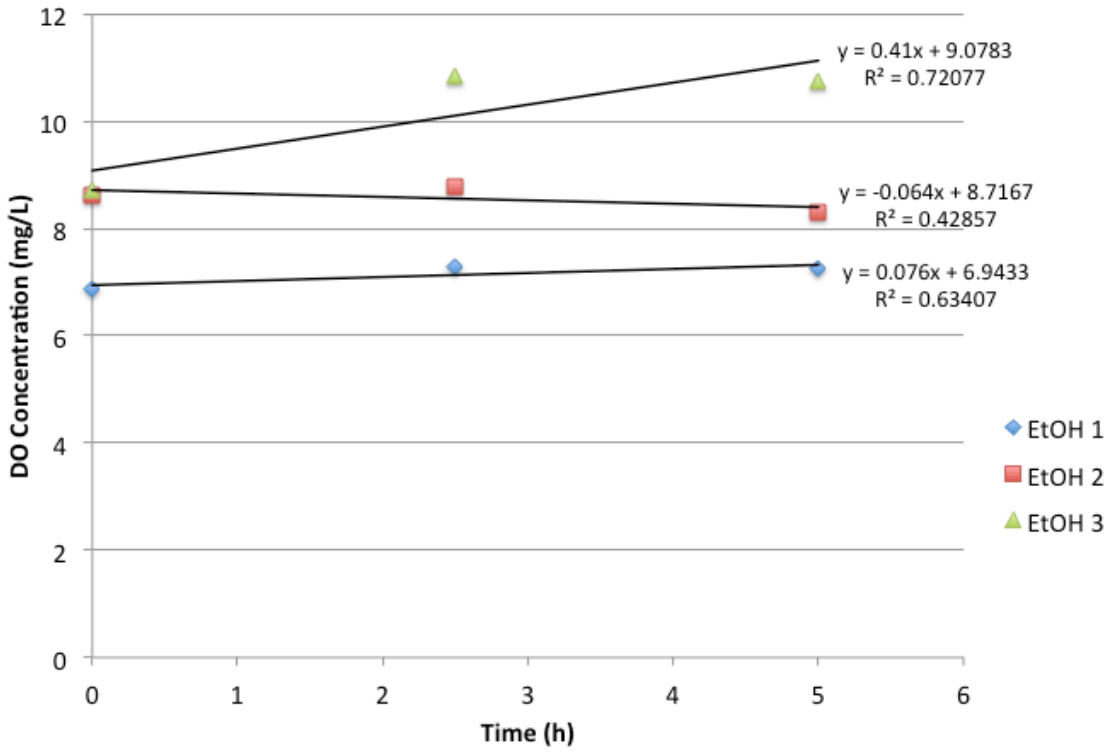



**Figure 3. Change in oxygen concentration over time for cells treated with ethanol**. Dissolved oxygen levels were measured at 0, 2.5, and 5 hours after addition of ethanol. Equations and $R^2$ values are directly to the right of the corresponding trendline. The rate of change in oxygen concentration was determined as the slope of the trendline. Culture concentrations were 1.75 X $10^6$ cells/ml for EtOH 1, 2.05 X $10^6$ cells/ml for EtOH 2, and 2.5 X $10^6$ cells/ml for EtOH 3.

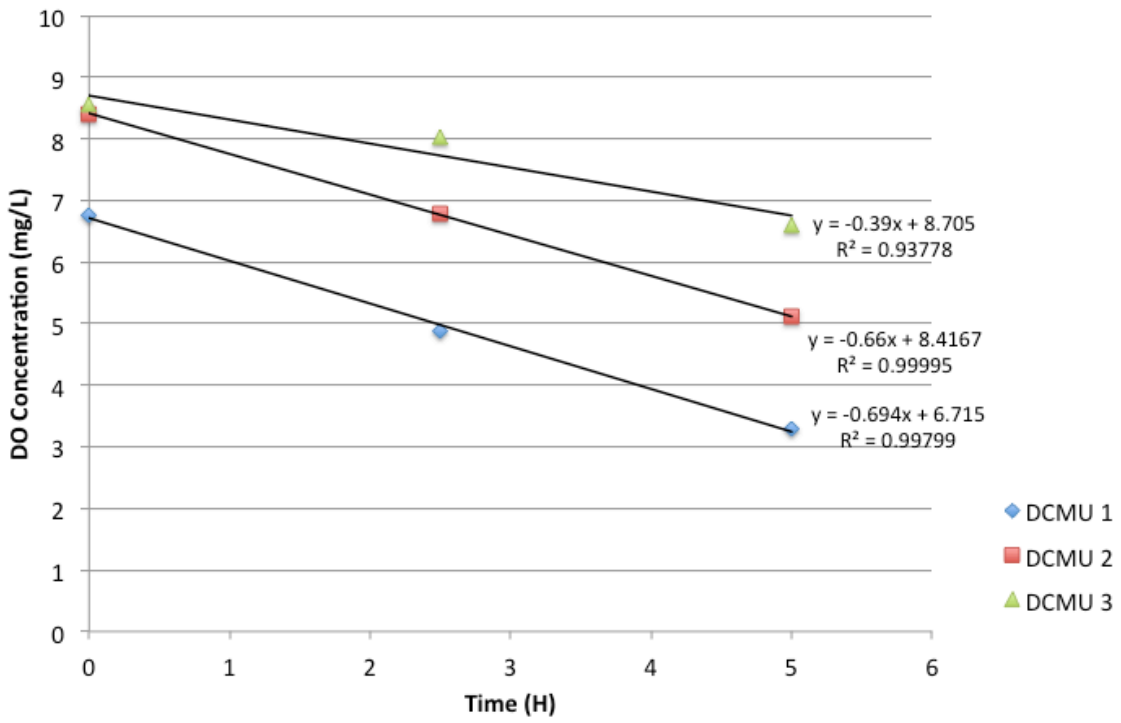



**Figure 4. Change in oxygen concentration over time for cells treated with DCMU**. Dissolved oxygen levels were measured at 0, 2.5, and 5 hours after addition of DCMU. Equations and $R^2$ values are directly to the right of the corresponding trendline. The rate of change in oxygen concentration was determined as the slope of the trendline. Culture concentrations were 1.75 X $10^6$ cells/ml for DCMU 1, 2.05 X $10^6$ cells/ml for DCMU 2, and 1.5 X $10^6$ cells/ml for DCMU 3.

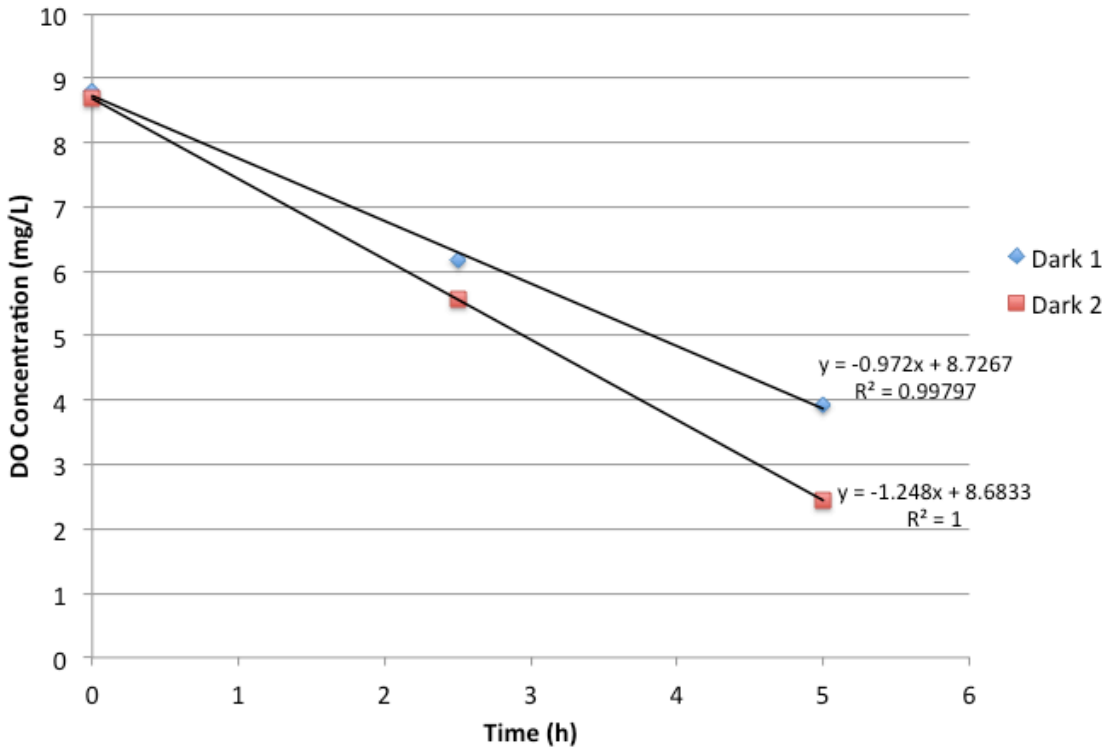



**Figure 5. Change in oxygen concentration over time for cells treated with ethanol and kept under dark conditions**. Dissolved oxygen levels were measured at 0, 2.5, and 5 hours after addition of ethanol. Equations and $R^2$ values are directly to the right of the corresponding trendline. The rate of change in oxygen concentration was determined as the slope of the trendline. Culture concentrations were 1.75 X $10^6$ cells/ml for Dark 1, and 2.5 X $10^6$ cells/ml for Dark 2.

receive a light pulse (Forbes-Stovall 2011). If photosynthesis were involved, the expected result would be that cells treated with DCMU would require a higher light intensity to achieve the same phase delay as cells without DCMU treatment. As shown in Figure 6, there was no difference in sensitivity between cells treated with DCMU and cells that were not. The result was confirmed by finding no significant difference between treatment conditions using an ANOVA ($p = 0.05$). This indicates that photosynthesis is not involved in resetting the clock upon blue light.

Screening transformants for cryptochrome knockdown

The previously described photoreceptor plant-like cryptochrome (Small et al. 1995) represents another possibility for how *C. reinhardtii* might detect blue light as a time cue when resetting its clock. To test this hypothesis, a former student designed an RNA interference (RNAi) construct to knockdown cryptochrome expression and transformed it into strain CC48. Strain CC48 was chosen because it carries a mutation causing arginine auxotrophy, which is reversed through the expression of the *C. reinhardtii* marker on the RNAi construct. In this study, transformants able to grow without exogenous arginine were screened for knockdown in cryptochrome expression using western blot analysis (Figure 7). The two strains cryRNAi16 and cryRNAi18 were found to have consistently reduced cryptochrome expression over several experiments, with expression relative to the parent strain of approximately 35% and 27%, respectively (Figure 8). Aliquots of these strains were frozen for long-term storage.

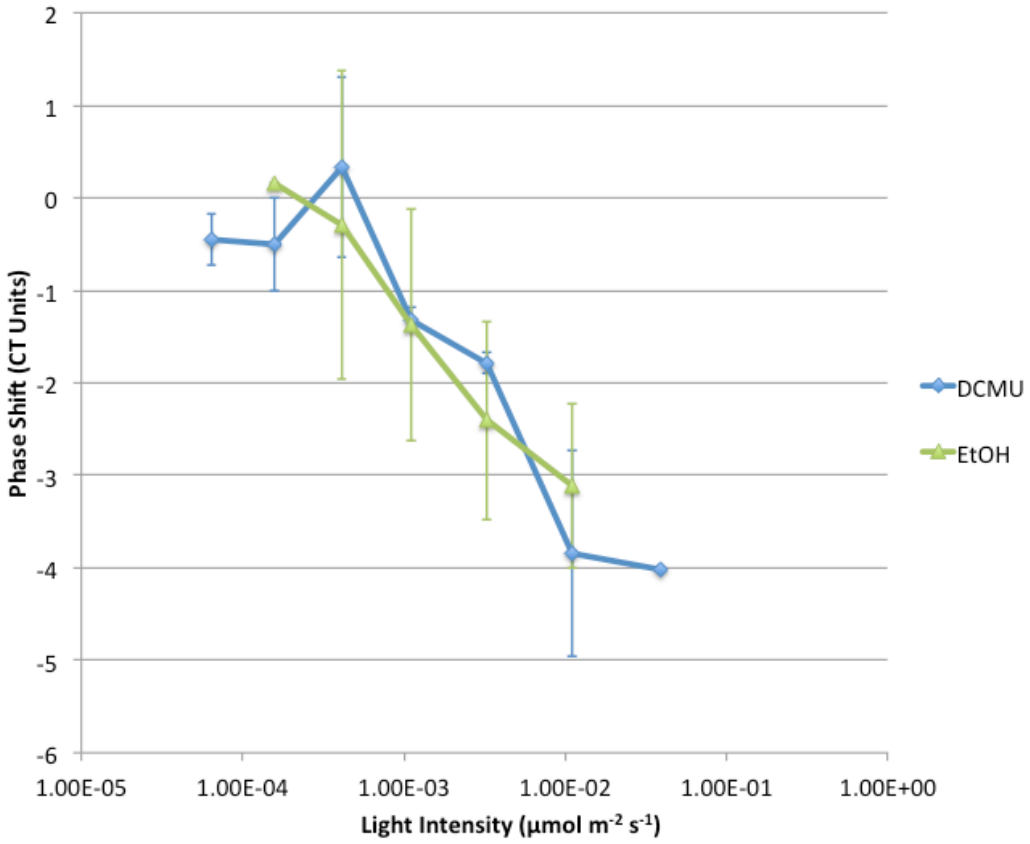



**Figure 6. Phase shifts in the presence of a photosynthesis inhibitor.** Fifteen-minute 440 nm light pulses were given at LD19 using a 2.0 neutral density filter. DCMU data points in the graph represent averages of 1-2 independent experiments. EtOH data points represent averages of 1-3 independent experiments. Error bars represent one standard deviation. A CT Unit represents 1/24 of the measured circadian period.

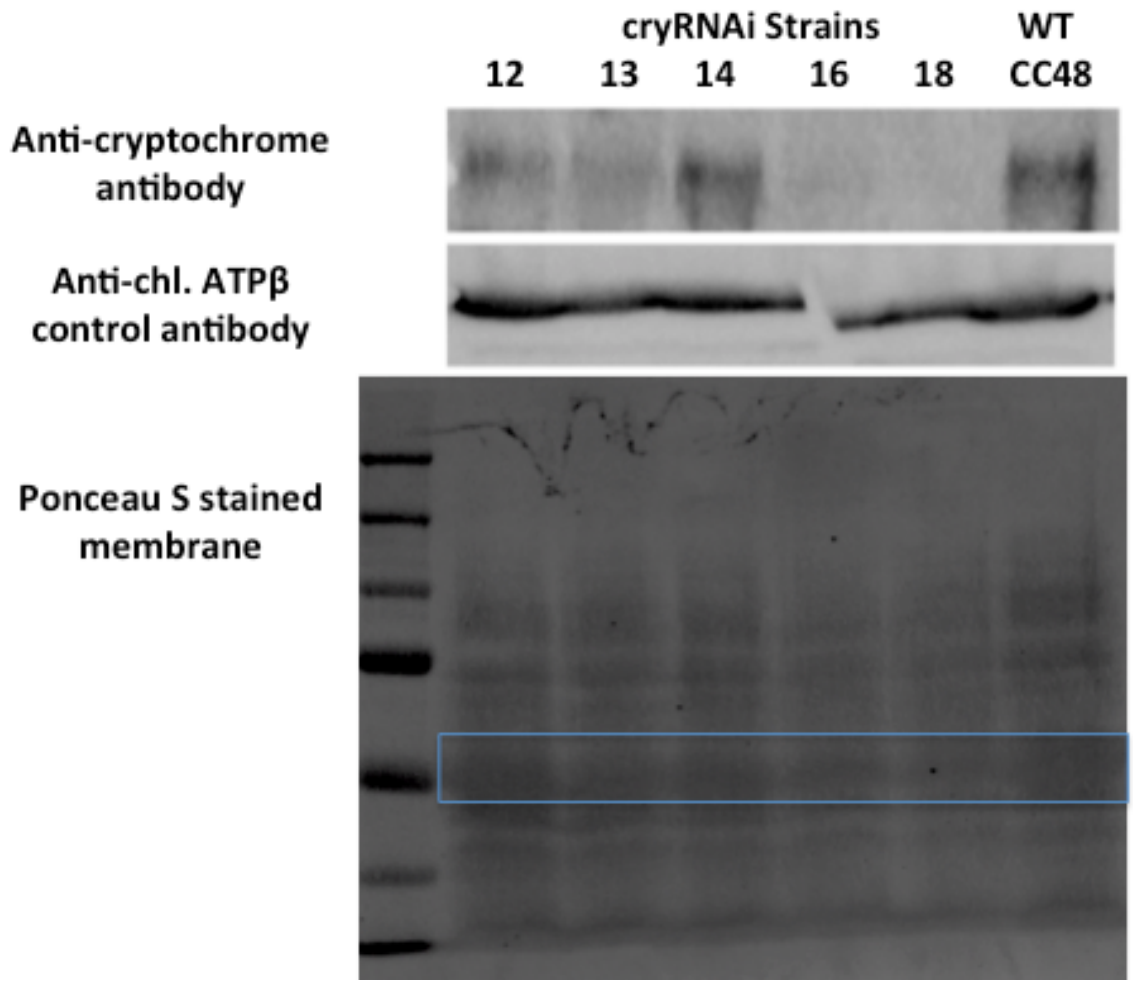



**Figure 7. Screening transformants of *C. reinhardtii* for cryptochrome knockdown using western blot analysis.** A representative western blot comparing cryptochrome expression between parent strain CC48 and RNAi transformants is shown (upper panel). In this particular experiment, 83.4 µg of total protein was loaded in each lane. Equal loading was confirmed using a control antibody against the beta subunit of the chloroplastic ATP synthase (middle panel) and by treating the membrane with the general protein stain Ponceau S before antibody incubation (lower panel). Box: band used as loading control to normalize calculated cryptochrome amounts.

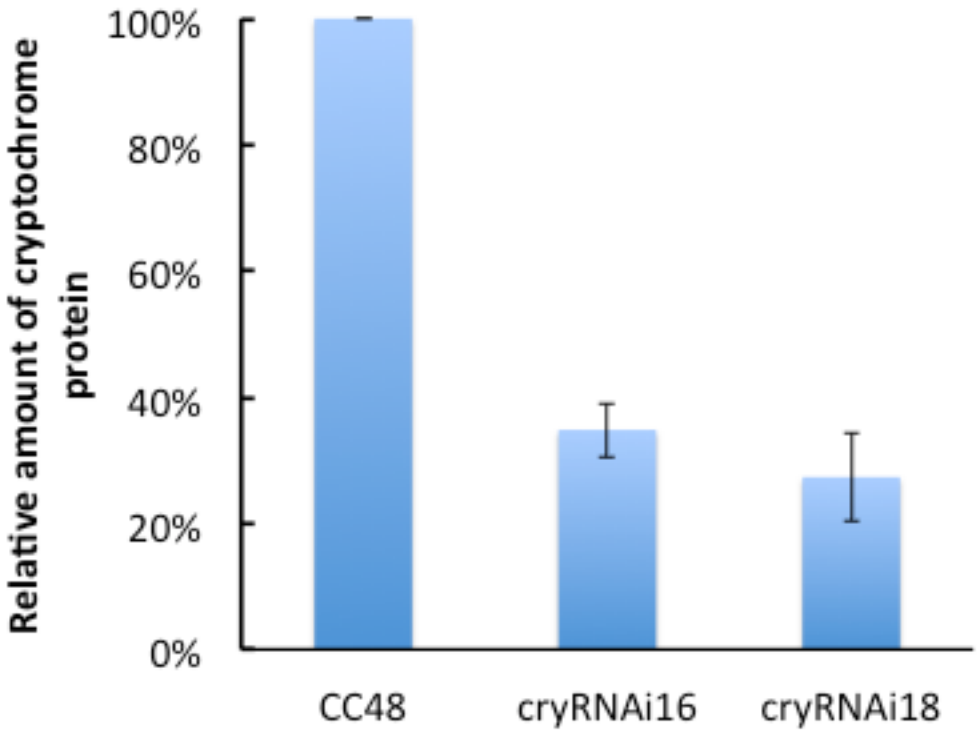



**Figure 8. Quantitative analysis of western blots as depicted in Figure 7**. The amount of cryptochrome protein in the RNAi transformants is expressed relative to the parent strain CC48, which was set as 100%. Data are normalized to the Ponceau S stained membranes, which served as loading controls. Columns represent averages of 3 independent experiments. Error bars represent one standard deviation.

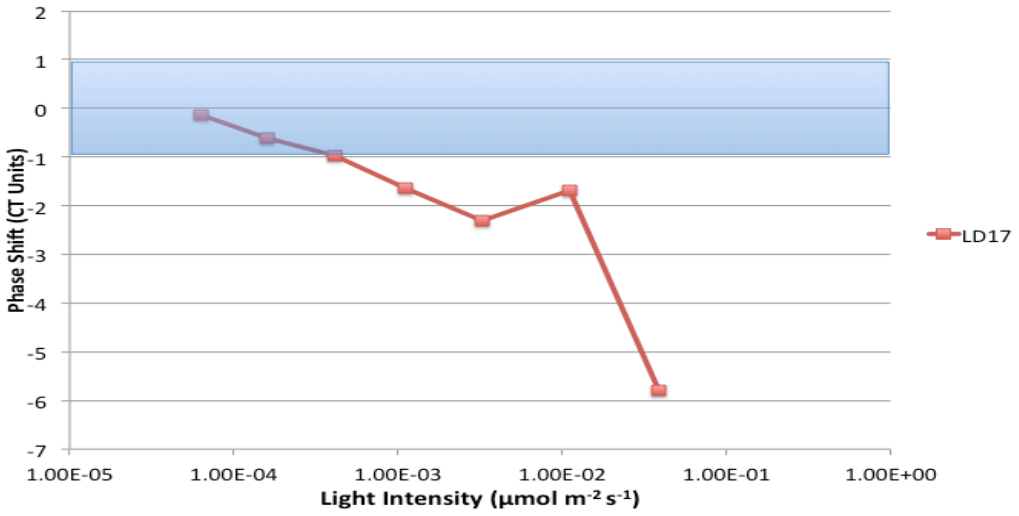
Optimization of light pulse

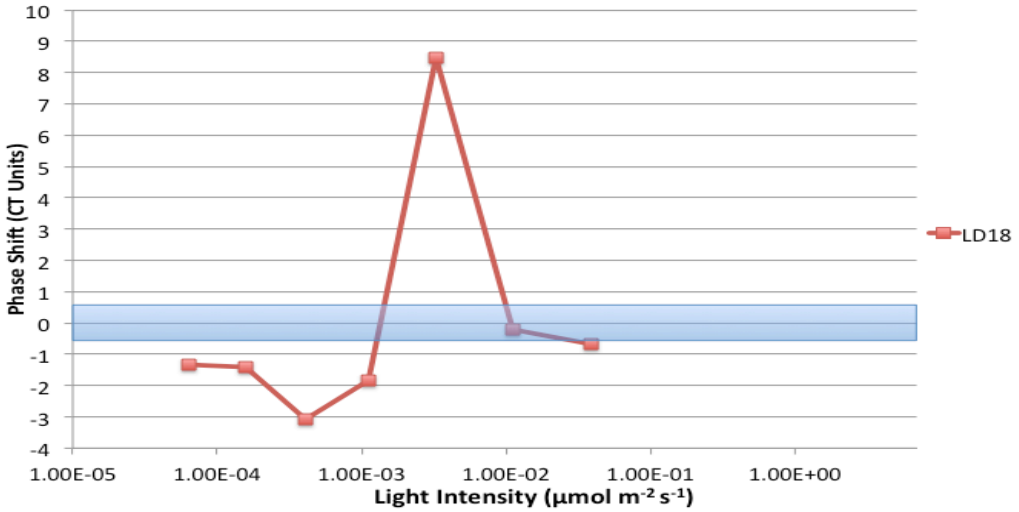
Before testing the RNAi strains for a possible defect in their ability to reset the clock upon blue light, the optimal time for giving the light pulse had to be determined. Blue light pulses were applied for 30 minutes using a 2.0 neutral density filter at LD17, LD18, and LD19 for the parent strain CC48 (Figure 9). At LD17 and LD19, a greater phase delay was measured as light intensity increased, with the lowest light intensities producing no phase shift (Figure 9). Thus, the intensity range caused a phase shift range that included the important threshold region. Light pulses at LD18 did not produce the same pattern (Figure 9). LD17 was chosen for future light pulse experiments, though LD19 appears to produce an equally useful phase shift pattern.

Additionally, a 15-minute light pulse seemed to be an improvement on the experimental design, as it would more precisely deliver a time cue to the culture at the designated time. Since this would result in less total light provided to the culture during the time point optimization, a 1.0 neutral density filter was used instead of the 2.0 because it would transmit more of the light beam to the culture. It was found that a 15-minute light pulse using the 1.0 neutral density filter (Figure 10) produced a phase shift pattern that was optimal by extending over the entire range of the response including the threshold region.

Cryptochrome knockdown reversion

When transformants cryRNAi16 and cryRNAi18 were investigated for their response to blue light pulses, the phase shifts they showed did not differ much from those of the parent strain (data not shown). One possible explanation for this finding is that the


2
1
0
-1
-2
-3
-4
-5
-6
-7
Phase Shift (CT Units)
1.00E-05
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
Light Intensity (µmol m-2 s-1)
LD17


10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
Phase Shift (CT Units)
1.00E-05
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
Light Intensity (µmol m-2 s-1)
LD18

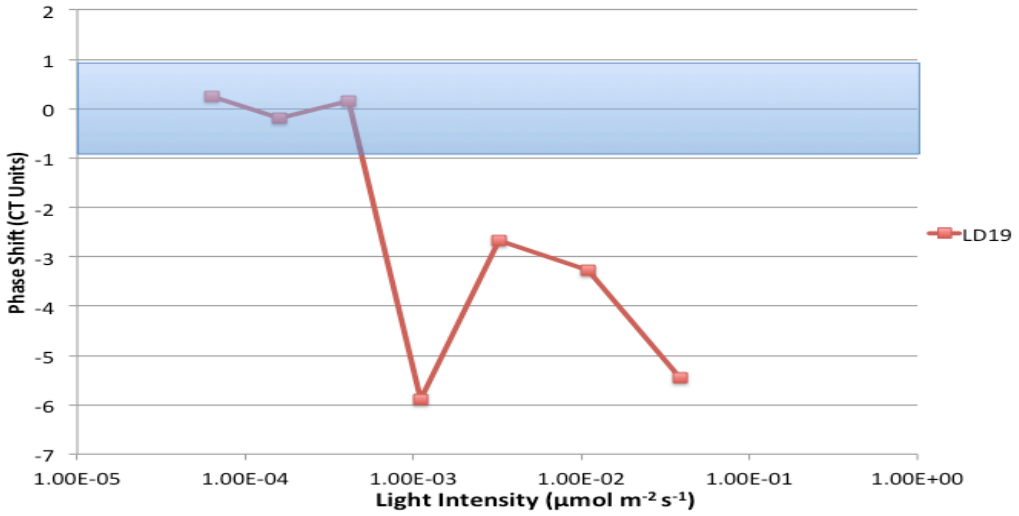

2
1
0
-1
-2
-3
-4
-5
-6
-7
Phase Shift (CT Units)
1.00E-05
1.00E-04
1.00E-03
1.00E-02
1.00E-01
1.00E+00
Light Intensity (µmol m-2 s-1)
LD19

**Figure 9. Optimization of light pulse time for strain CC48.** Thirty-minute light pulses were given using a 440nm bandpass filter and a 2.0 neutral density filter at time points LD17 (Top Panel), LD18 (Middle Panel), and LD19 (Bottom Panel), which is 5, 6, and 7 hours into the dark phase of a 12 h light/12 h dark cycle, respectively. Plotted phase shifts are the result of a single experiment. The shaded areas represent the phase shift range of dark controls. A CT Unit represents 1/24 of the measured circadian period.

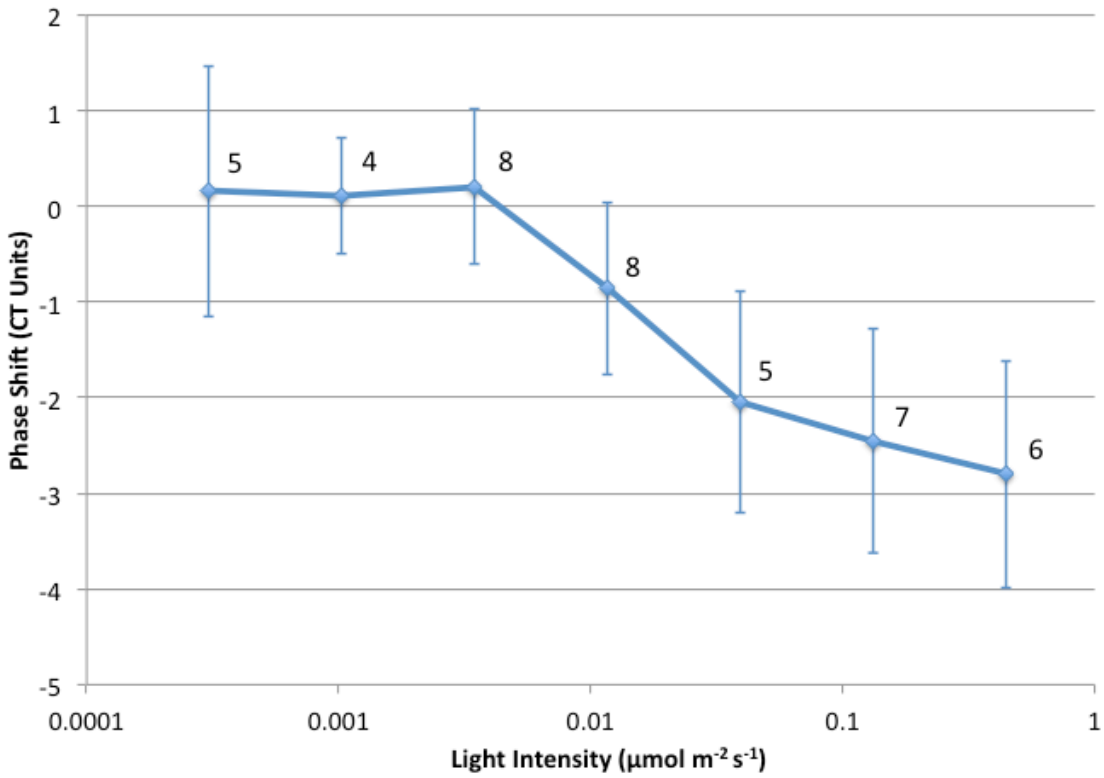



**Figure 10. Phase shifts of the parent strain CC48 using a 1.0 neutral density filter and a 15 min light pulse**. Light pulses were given at LD17 using a 440 nm bandpass filter. The data points in the graph represent the average of several independent experiments. Numbers beside the data points represent the exact number of independent experiments that were performed for the data point. Error bars represent one standard deviation. A CT Unit represents 1/24 of the measured circadian period.

expression of cryptochrome had reverted to wild-type levels, which is a phenomenon previously reported for *C. reinhardtii* that is due to its ability to silence transgenes (Rohr et al. 2004; Yamasaki et al. 2008). To determine if cryptochrome expression was still reduced at the time of the light pulse, western blot analysis was performed on samples obtained directly at the time of a light pulse (Figure 11). It was found that the transformants had not only reverted from the RNAi knockdown effect, but were actually expressing a larger amount of cryptochrome protein relative to the parent strain CC48 (Figure 12). Compared to CC48, strain cryRNAi18 showed about twice as much and strain cryRNAi16 had about four times as much.

Frozen stocks retained cryptochrome knockdown

As aliquots of the strains cryRNAi16 and cryRNAi18 were frozen immediately after screening, there was the possibility that they still showed RNAi knockdown after thawing. Accordingly, western blot analyses were performed on the thawed strains. As demonstrated with the example in Figure 13, both cryRNAi16 and cryRNAi18 still showed reduced amounts of cryptochrome relative to the parent strain. They expressed approximately 24% and 46%, respectively, relative to the parent strain (Figure 14).

Testing ability to reset clock in cryRNAi strains

During each time when a sample of the thawed cryRNAi strains was collected for western blot analysis, replicate samples were exposed to blue light pulses to determine their ability of shifting the phase of their circadian phototaxis rhythm (Figure 15). The strain cryRNAi16 was found to be significantly more sensitive than the parent strain

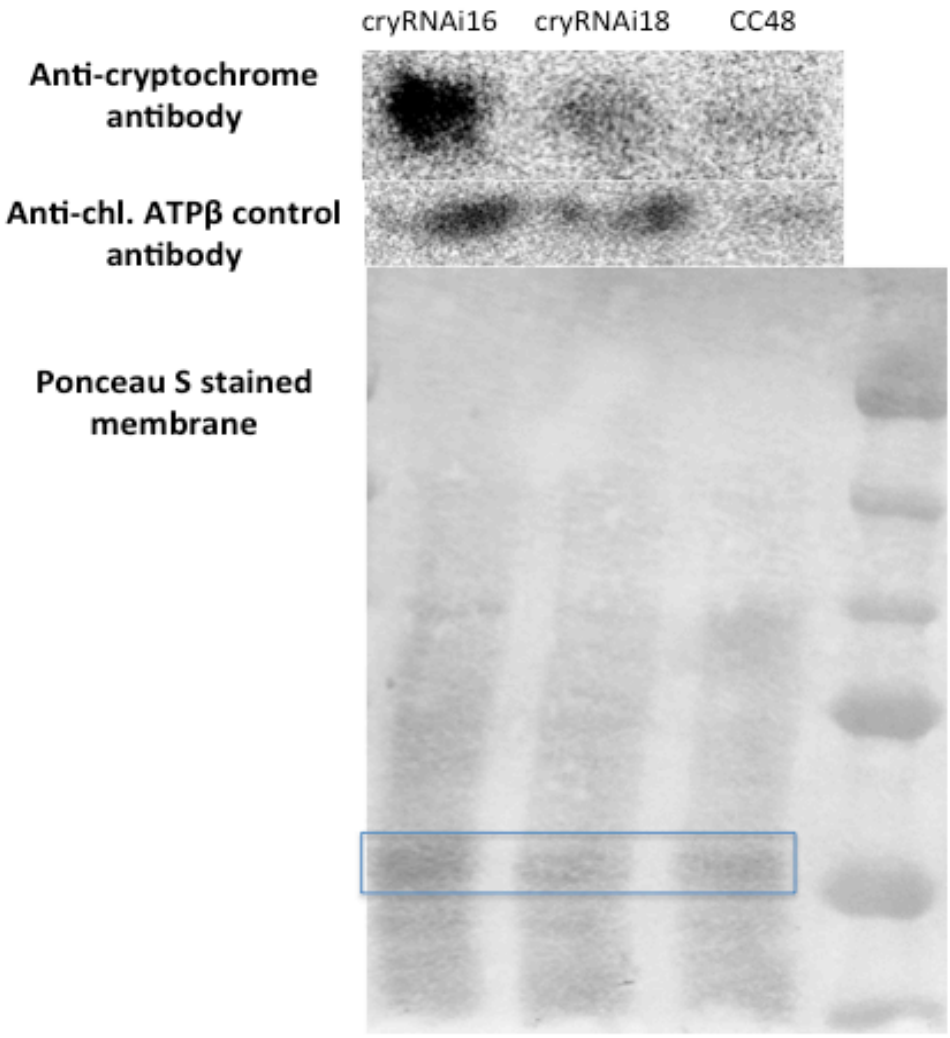



**Figure 11. Cryptochrome amounts at time of light pulse.** A representative western blot comparing cryptochrome expression between parent strain CC48 and RNAi transformants is shown (upper panel). In this particular experiment, 64.2 µg of total protein was loaded in each lane. Equal loading was confirmed using a control antibody against the beta subunit of the chloroplastic ATP synthase (middle panel) and by treating the membrane with the general protein stain Ponceau S before antibody incubation (lower panel). Box: band used as loading control to normalize calculated cryptochrome amounts.

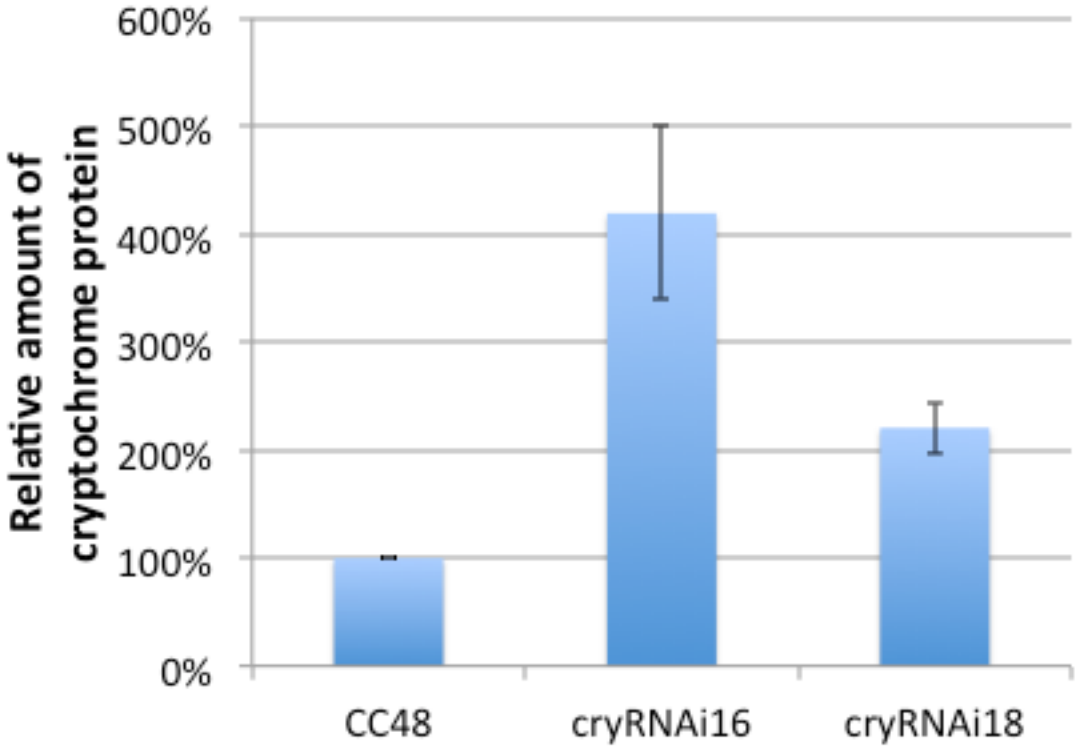



**Figure 12. Quantitative analysis of western blots as depicted in Figure 11.** The amount of cryptochrome protein present in the RNAi transformants is expressed relative to the parent strain CC48, which was set at 100%. Data are normalized to the Ponceau S stained membranes, which served as loading controls. Columns represent averages of 2 independent experiments. Error bars represent one standard deviation.

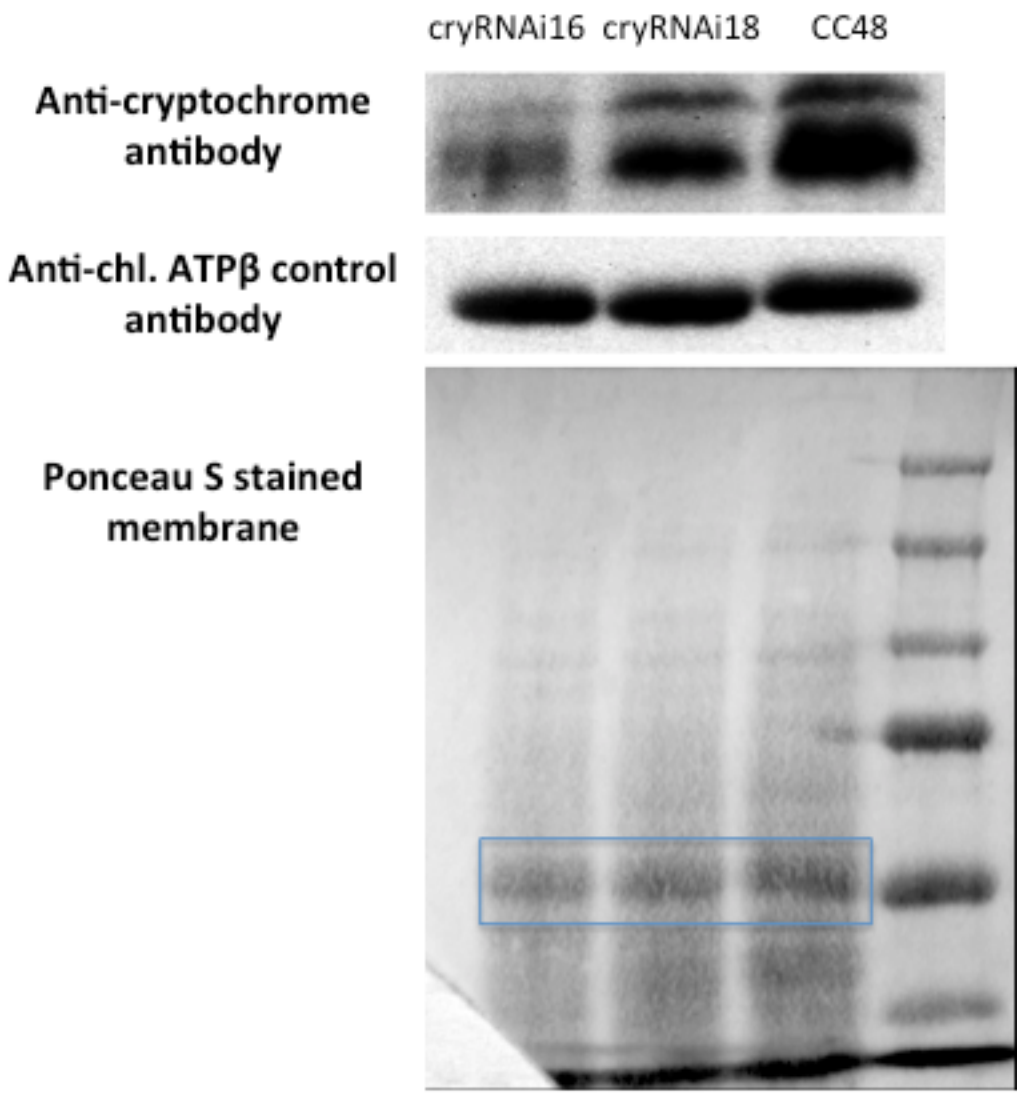



**Figure 13. Cryptochrome amounts at time of light pulse for cryRNAi strains recovered from frozen storage.** A representative western blot comparing cryptochrome expression between parent strain CC48 and RNAi transformants is shown (upper panel). In this particular experiment, 87.4 µg of total protein was loaded in each lane. Equal loading was confirmed using a control antibody against the beta subunit of the chloroplastic ATP synthase (middle panel) and by treating the membrane with the general protein stain Ponceau S before antibody incubation (lower panel). Box: band used as loading control to normalize calculated cryptochrome amounts.

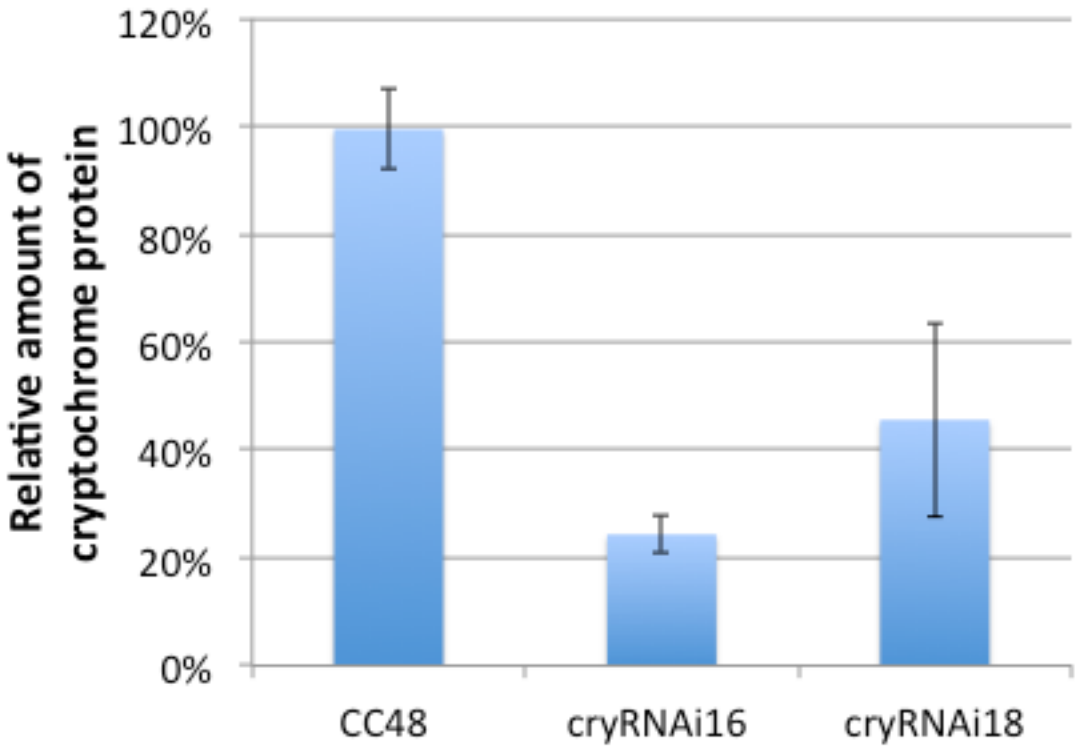



**Figure 14. Quantitative analysis of western blots as depicted in Figure 13.** The amount of cryptochrome protein present is relative to the average of two parent strain CC48 samples per western blot membrane, which was set at 100%. Data are normalized to the Ponceau S stained membranes, which served as loading controls. Columns represent averages of 4 independent experiments. Each independent experiment is the average of 3-4 technical replicates. Error bars represent one standard deviation.

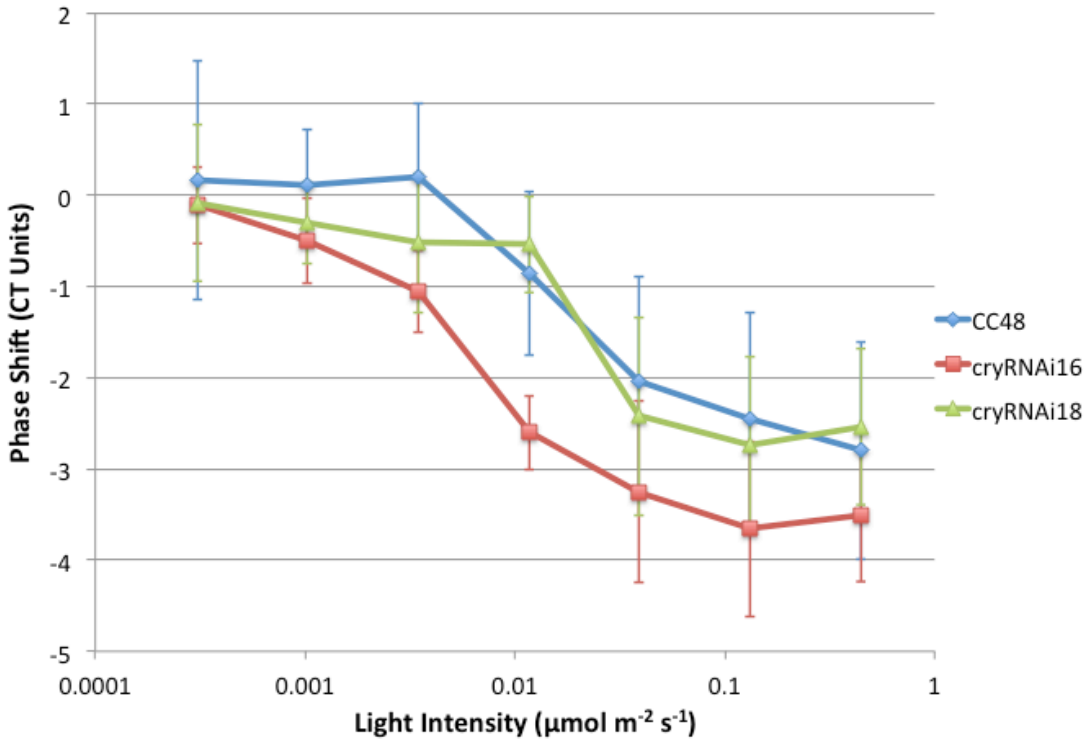



**Figure 15. Phase shifts of cryRNAi strains after recovering from frozen storage as compared to the parent strain CC48.** Fifteen-minute 440nm light pulses were given using a 1.0 neutral density filter. All data points are the mean of at least 3 independent experiments. Error bars represent one standard deviation. A CT Unit represents 1/24 of the measured circadian period.

CC48 using an analysis of variance (ANOVA). This was surprising because the expectation had been that a reduced expression of cryptochrome would cause a strain to be less sensitive. CryRNAi16 was also found to be significantly more sensitive than cryRNAi18, but CC48 and cryRNAi18 were not significantly different from one another.

Strains lost cryptochrome knockdown after second freeze

Cultures were also obtained from stocks that had been refrozen immediately after the first set of frozen stocks was thawed. Based upon western blot analysis, these cryRNAi cultures reverted from the cryptochrome knockdown state to equal or greater cryptochrome expression relative to the parent strain (Figure 16). In fact, the pattern seemed similar to the one obtained for the revertants that had never been frozen (Figure 11), because cryRNAi16 showed a much higher amount of cryptochrome than the parent strain, whereas cryRNAi18 showed an amount higher than the parent strain but not as high as cryRNAi16. However, when cryptochrome transcript levels were determined using qRT-PCR, they showed a trend indicative of persistant cryptochrome knockdown in the cryRNAi strains, though there was considerable variation in the measurements (Figure 17). Both cryRNAi strains showed phase shift response curves that were very similar to the parent strain (Figure 18), and no significant difference was found between strains using an ANOVA.

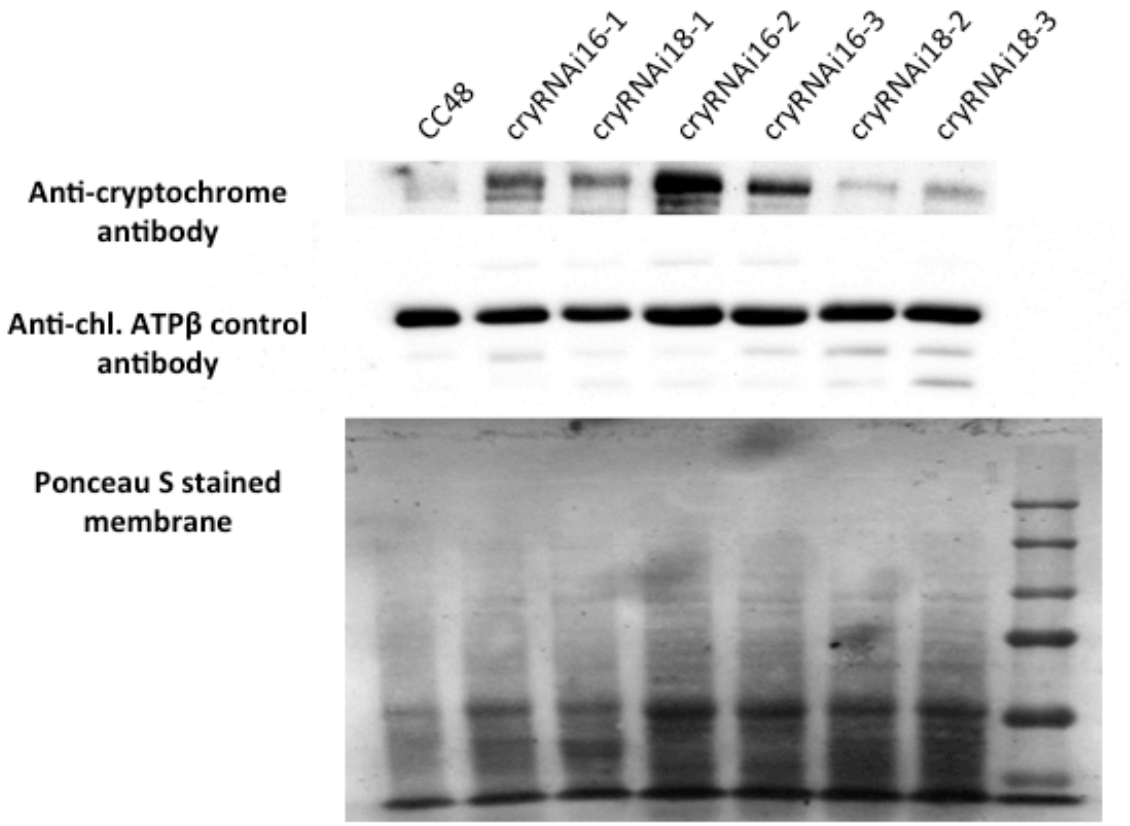



**Figure 16. Cryptochrome amounts at time of light pulse for cryRNAi strains that had been frozen a second time.** A representative western blot comparing cryptochrome expression between parent strain CC48 and RNAi transformants is shown (upper panel). In this particular experiment, 88.1 μg of total protein was loaded in each lane. Equal loading was confirmed using a control antibody against the beta subunit of the chloroplastic ATP synthase (middle panel) and by treating the membrane with the general protein stain Ponceau S before antibody incubation (lower panel).

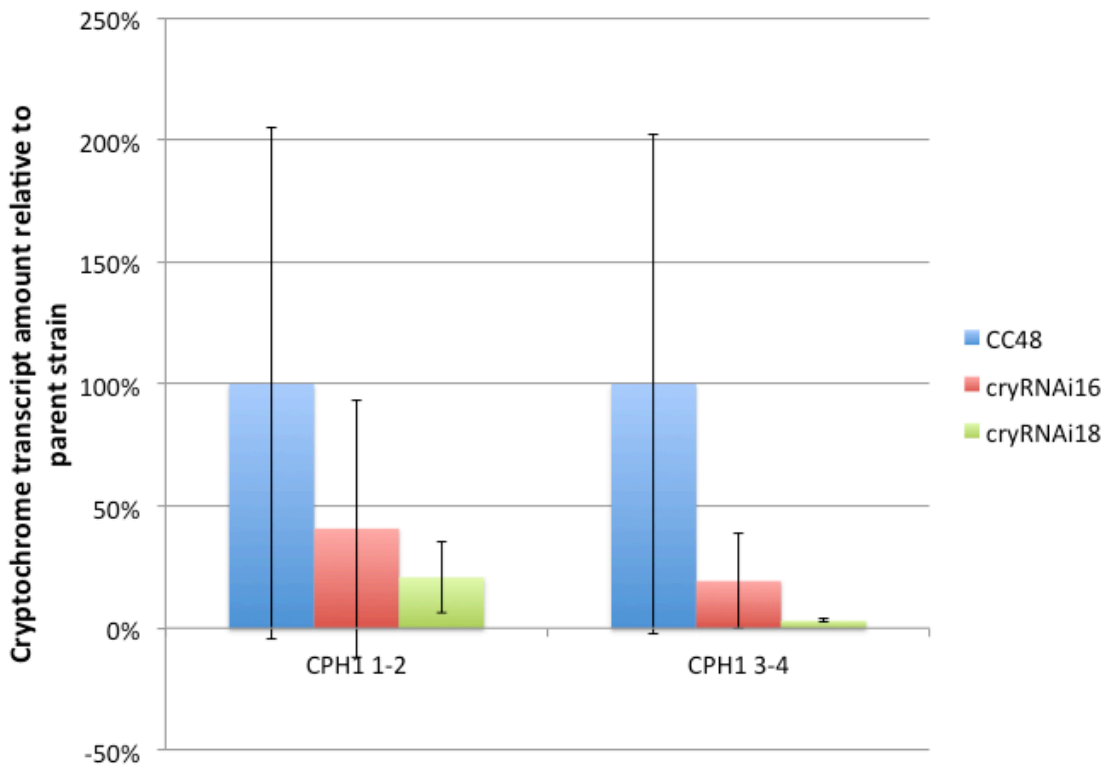



**Figure 17. Quantitative Real Time PCR analysis at time of light pulse for cryRNAi strains that had been frozen a second time.** The amount of cryptochrome mRNA present is expressed with respect to the amount of 18S rRNA. It is further expressed relative to the parent strain CC48, which was set at 100%. Primers are named for the exon-exon boundary in the cryptochrome (CPH1) cDNA. CPH1 1-2 overlaps the exons 1/exon 2 boundary, and CPH1 3-4 overlaps the exon 3/exon 4 boundary. CC48 columns represent averages of 4 independent experiments, while cryRNAi16 and cryRNAi columns represent averages of 3 independent experiments. Error bars represent one standard deviation.

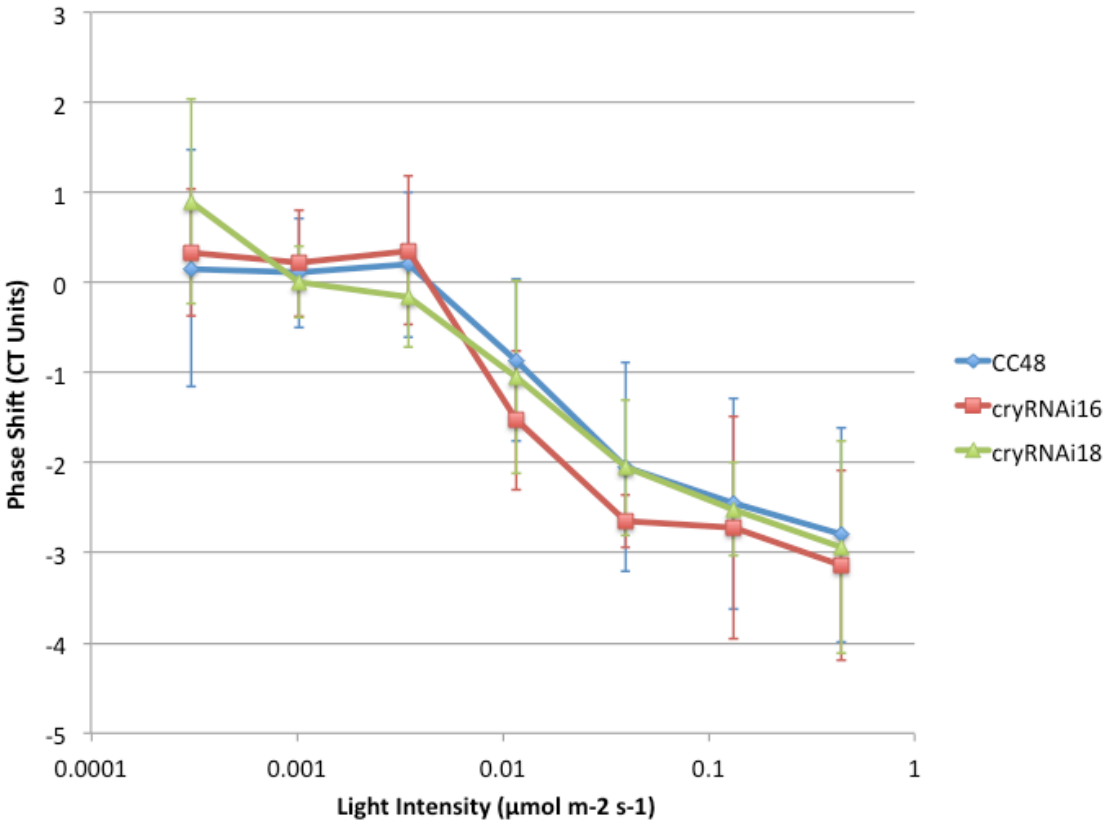



**Figure 18. Phase shifts of cryRNAi strains that had been frozen a second time as compared to the parent strain CC48.** Fifteen-minute 440nm light pulses were given using a 1.0 neutral density filter. All data points are the mean of at least 4 independent experiments. Error bars represent one standard deviation. A CT Unit represents 1/24 of the measured circadian period.

# CHAPTER IV

## Discussion

This study was designed to determine the kind of molecule that perceives blue light as a time cue to reset the circadian clock in *C. reinhardtii*. It first focused on the question whether photosynthesis mediates the resetting through its blue light absorption by chlorophylls and carotenoids, or whether the response is due to a photoreceptor.

Using an inhibitor of photosynthetic electron transport, it was determined that photosynthesis is not involved in the blue light induced photoentrainment. Phase shifts were nearly identical in the presence and in the absence of the inhibitor (Figure 6). This result was expected, as the light intensities of the light pulses inducing the phase shifts are much lower than what is typically required for responses mediated by photosynthesis. The higher light intensity is required because the amount of photosynthesis that occurs is directly proportional to the amount of light energy that is absorbed. Therefore, it requires much more energy than a photoreceptor molecule, which amplifies the signal. For example, photosynthesis had previously been found to be involved in red and blue light induced resetting of the clock in light-adapted cell wall deficient *C. reinhardtii* mutant strain CW15 kept in constant dim light (Johnson et al. 1991), but not in cells kept in constant darkness (Kondo et al. 1991). Compared to this study, the blue light intensity used by Johnson and colleagues (1991) for the photosynthesis-mediated resetting was much greater (50 μmol $m^{-2}$ $s^{-1}$ versus 0.039 μmol $m^{-2}$ $s^{-1}$ or less) and light pulses lasted 24-fold longer (6 hours versus 15 min). In this study, only 66% of photosynthesis blocked through treatment with the inhibitor, so it is possible that the negative result is due to insufficient photosynthesis inhibition. However, it was previously shown in light-

adapted cells that blocking only 33% of photosynthesis completely eliminated phase shifting (Johnson et al. 1991). So, blocking twice as much of the photosynthesis activity should definitely block blue light-induced phase shifting if photosynthesis mediates this response. Therefore, photosynthesis may be involved in blue light induced resetting of the clock in *C. reinhardtii* at high light intensities, but a much more sensitive photoreceptor must be involved in resetting the clock upon lower intensity blue light.

The blue light sensing photoreceptor plant-like cryptochrome was a likely candidate for mediating clock resetting, as cryptochrome has been shown to be involved in resetting the clock in *Arabidopsis* (Somers et al. 1998). In order to test cryptochrome's involvement in this process, a previous student designed an RNAi construct (Figure 19) to knockdown cryptochrome expression and transformed this construct into strain CC48. CC48 was chosen as the recipient strain because it is unable to produce arginine due to a mutation in the argininosuccinate lyase gene, and the RNAi construct contained a *C. reinhardtii* marker that reversed this arginine auxotrophy. This allowed transformants to be selected for their ability to grow in media without arginine.

In this study, the transformants cryRNAi16 and cryRNAi18 were originally found to have consistently reduced cryptochrome expression through initial western blot screenings. However, western blot analysis of samples taken at a later time showed that cryptochrome expression was no longer reduced. It even demonstrated that the amount was actually greater than that found in the parent strain (Figures 8 and 9). It is likely that loss of cryptochrome knockdown was caused by a commonly described phenomenon in

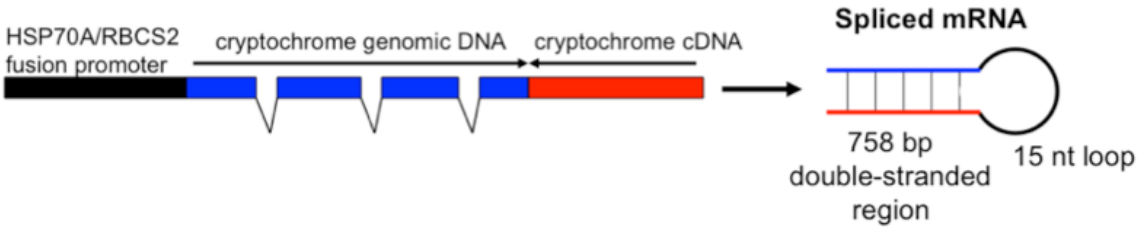



**Figure 19. Stucture of cryptochrome RNA interference gene and the double-stranded mRNA it leads to when expressed in *C. reinhardtii*.** The RNA interference gene consists of a *C. reinhardtii* promoter fused to a fragment of the cryptochrome genomic DNA, which is followed by a complimentary piece of cryptochrome cDNA in reverse orientation. When this gene is expressed and processed into mRNA, it forms a 758 bp double-stranded region with a 15-nucleotide loop connecting the two regions.

*C. reinhardtii,* in which the transgene is silenced (Rohr et al. 2004; Yamasaki et al. 2008). In this study, the construct consisted of a piece of cryptochrome genomic DNA followed by a complementary piece of cryptochrome cDNA in reverse orientation so that the mRNA transcribed would form a double stranded RNA by folding back upon itself after processing (Figure 19). This method of using a single inverted repeat was developed for use in *C. reinhardtii* by Furhmann and colleagues (2001) and has been successfully adapted for targeting a broad array of transcripts (Schroda 2006), but it has no means to prevent transgene silencing. This problem can now be avoided by using the recently developed tandem inverted repeat RNAi system, in which a second inverted repeat designed to target the Maa7 gene is used to allow transformants to grow in otherwise toxic media; the Maa7 inverted repeat is fused to the inverted repeat designed to target the gene of interest so that they are always expressed together (Rohr et al. 2004). This provides selective pressure to maintain expression of both inverted repeats within the construct, and leads to a more stable knockdown of the target gene. (Kim and Cerutti 2009). Using this technique, RNAi induced knockdown is also less variable between transformants. Another recently developed alternative RNAi method is the use of artificial micro RNA (amiRNA) in *C. reinhardtii*. The technique has been shown to not only reduce the phenomenon of trans-gene silencing, but also to reduce the amount of off-target effects by producing a predictable amiRNA that can be designed so that it is only complimentary to a region within the target transcript (Molnar et al. 2009). The latter is a benefit over using an inverted repeat system because the double-stranded RNA produced by the inverted repeat is cleaved unpredictably. Additionally, the amiRNA strategy has been modified to allow for two independent amiRNAs to be produced from a

single amiRNA precursor so that one could be used as a marker to supply selective pressure for the production of the amiRNAs by targeting Maa7 expression as with the tandem inverted repeat system (Zhao et al. 2009). A very useful third alternative would be to produce a knockout mutant, because this would eliminate off-target effects and would provide a consistent state in which cryptochrome is completely absent. Unfortunately, targeted gene knockouts have been very difficult to produce in *C. reinhardtii* due to its low frequency of homologous recombination. Very recently, though, a technique has been optimized to produce a knockout mutant for a target gene using the random insertion of an APHVIII gene cassette and a moderate-throughput PCR based screening process (Gonzalez-Ballester et al. 2011). This technique has just been used to characterize an animal-like cryptochrome in *C. reinhardtii* (Beel et al. 2012), so it should be possible to apply it to study plant-like cryptochrome as well. Lastly, while the trans-gene silencing could explain why cryptochrome expression was no longer knocked down, it is still unclear why the amount was actually greater than that of the parent strain or why mRNA levels appeared to still be reduced despite these higher amount (Figures 17 versus 16).

Cultures made from cryRNAi stocks frozen immediately after the initial screening still showed cryptochrome knockdown. When these strains were tested for their ability to reset their circadian clock upon blue light, it was found that they were not less sensitive than the parent strain (Figure 15). Thus plant-like cryptochrome is probably not the photoreceptor that promotes this response. On the contrary, an ANOVA confirmed that cryRNAi16 was significantly more sensitive to blue light induced photoentrainment than the parent strain. This was not found for cryRNAi18, which did not show a significant

difference to the parent strain. Since cryRNAi16 had lower cryptochrome expression than both the parent strain and cryRNAi18, it is possible that this increase in sensitivity is a direct result of the lower cryptochrome amount. It means that cryptochrome may influence the blue light response by acting through an inhibitory mechanism that modulates the function of another photoreceptor. A mechanism such as this has been described before. The blue light photoreceptor VIVID performs a function like this in the fungus *Neurospora*; it is a circadianly expressed protein that regulates the sensitivity of the input pathway by decreasing the transcript, phosphorylation, and protein levels of WHITE COLLAR-1, which acts as the photoreceptor of the input pathway in *Neurospora* (Heintzen et al. 2001). Cryptochrome has not been tested to determine if it is circadianly expressed, but it has been shown to be expressed with a diurnal pattern (Reisdorph and Small 2004). This could allow cryptochrome to rhythmically regulate the blue light sensitivity of the input pathway in *C. reinhardtii* as VIVID does in *Neurospora*. To confirm this increase in sensitivity is truly an effect of reduced cryptochrome expression, another cryRNAi mutant would need to show the same response. It would then be more likely that the effect is independent of any secondary event. Another possibility for the increase in sensitivity is an off-target effect caused either by the location in which the construct integrated into the genome, or by the produced siRNAs affecting the expression of another transcript through partial complementarity. Additionally, the increase in sensitivity could also result from the overexpression of another blue light photoreceptor that functions as the true photoreceptor for resetting. A reduced amount of cryptochrome may be able to trigger a compensatory mechanism resulting in the increased expression of this other photoreceptor. Regardless, this study supports the conclusion that plant-like

cryptochrome is not the photoreceptor that promotes clock resetting in *C. reinhardtii* upon blue light. This is interesting because resetting appeared to be the most likely function of this particular cryptochrome in *C. reinhardtii* as it shows an amino acid sequence that is very similar to *Arabidopsis* cryptochromes (Small et al. 1995), which clearly exhibit this function (Somers et al. 1998).

In general, a negative result based upon RNAi strains cannot be completely conclusive. This is a general weakness of using the RNAi technique because it only reduces the amount of protein expressed. In this case, there was still cryptochrome present to detect the light pulse. Because photoreceptors are involved in signal transduction pathways that amplify signals, the signal produced could still be quite large even though cryptochrome expression is much lower. This would likely occur if cryptochrome responded to light in an all-or-nothing threshold manner (i.e. either on or off), as apposed to a graded manner in which the amount of signal received directly determines the amount of response. The first instance is known to occur, for example, with the closing of a Venus flytrap; this plant closes on its prey immediately after two touches of its trigger hairs (Affolter and Olivo 1975). The plant is either open or closed, and the extent to which it closes is not directly related to the intensity or frequency of the stimulus. A graded response is exemplified by the phototropin photoreceptor in *C. reinhardtii*; it was demonstrated that both the amount of light absorbed by phototropin and the amount of phototropin remaining in RNAi strains were directly proportional to the amount gametogenesis it induced (Huang and Beck 2003). In this study the phase shifting clearly occurred as a graded response, so it would be expected that cryRNAi16

would be approximately 75% less sensitive than the parent strain as a result of its reduced expression of plant-like cryptochrome.

There are several other blue light photoreceptors in *C. reinhardtii* that could perform the function of mediating clock resetting. First of all, an animal-like cryptochrome (aCry) was found through homology searches of the *C. reinhardtii* genome (Mittag et al. 2005). This photoreceptor has recently been shown to regulate expression of many genes, including core clock components, upon absorption of blue and even red light (Beel et al. 2012). Because of this, aCry may be the true photoreceptor responsible for resetting the clock upon blue light. Another blue light photoreceptor found in *C. reinhardtii* is phototropin. It is a membrane-associated protein (Huang et al. 2002) found in both the cell body and the flagella (Huang et al. 2004). It has been shown to regulate the conversion of progametes to gametes (Huang and Beck 2003). Phototropin protein expression is circadianly regulated in *Arabidopsis* (Harmer et al. 2000), but there have been no tests to determine if this occurs in *C. reinhardtii*. Though phototropin detects blue light, it has not yet been tested if it is involved in photoentrainment of the clock in *C. reinhardtii*, but *Arabidopsis* phototropin1 is not involved in this process (Hamer et al. 2000). Another known blue light photoreceptor in this alga is the opsin-like protein channelrhodopsin2 (Nagel et al. 2003). This protein was shown to function in light sensing for phototaxis (Sineshchekov et al. 2002), but it has not been tested for any function within the circadian clock. However, Sineshchekov et al. (2002) showed that the wavelength of maximum phototactic response mediated by the protein is 470 nm, which differs from the 440 nm wavelength found to cause maximum action for resetting the clock in *C. reinhardtii* (Forbes-Stovall 2011).

Additionally, there is a putative blue light photoreceptor that has been found through homology searches of the *C. reinhardtii* genome. It is a homolog for a blue light photoreceptor found in both the green alga *Ostreococcus tauri* and in a diatom; this protein contains a LOV (light, oxygen, voltage sensing) domain like phototropin, but has a histidine kinase domain instead of the serine/threonine kinase domain found in phototropin (Djouani-Tahri et al. 2011). In *O. tauri*, this LOV-Histidine Kinase (LOV-HK) protein is circadianly regulated, and is an essential component of the circadian clock that acts independently of its blue light photoreceptor activity (Djouani-Tahri et al. 2011). This photoreceptor has not been tested to determine if it is involved in the input pathway in *O. tauri* or if its homolog is involved in the circadian clock of *C. reinhardtii*.

In addition, the input pathway in *C. reinhardtii* may be very complex like it is in *Arabidopsis*, where the input pathway involves the interaction between multiple photoreceptors, because green algae are very closely related to higher plants. In *Arabidopsis*, multiple photoreceptors are used to regulate clock input so that the plant can respond to light conditions in the environment that vary greatly in intensity and spectral quality. Phytochrome A is responsible for detecting low fluence red and blue light, while phytochrome B is responsible for detecting high fluence red light (Somers et al. 1998). Cryptochrome 1 and 2 are also involved and have redundant functions of retaining circadian period length at intermediate fluence rates of blue light, while at higher fluence rates of blue light, cryptochrome 1 acts alone and cryptochrome 2 is degraded (Somers et al. 1998). Additionally, cryptochrome 1 and phytochrome A are required to interact for responding to low fluence blue (Somers et al. 1998) and red light (Devlin and Kay 2001). If there is a complex interaction of photoreceptors like this in *C. reinhardtii*, then it

would require knockdown or knockout of multiple photoreceptors simultaneously to understand it fully.

This study supports the notion that a photoreceptor is responsible for mediating the blue light-induced entrainment of the circadian clock in *C. reinhardtii.* Furthermore, it shows that plant-like cryptochrome does not perform this function. Additionally, this study provides some indication that cryptochrome may be involved in this process in an inhibitory way. However, due to the limitations of the methods used, these results are not entirely conclusive. Therefore, future work might focus on obtaining true knockout mutants to allow for more unambiguous experiments. Furthermore, there are several other blue light photoreceptors in *C. reinhardtii* that should be investigated, among them particularly the animal-like cryptochrome.

REFERENCES

Affolter, J.M. and Olivo, R.F. (1975). Action Potentials in Venus's-Flytrap: longterm observation following capture of prey. *American Midland Naturalist*, 93, 443-445.

Beel, B., Prager, K., Spexard, M., Sasso, S., Weiss, D., Müller, N., Heinnickel, M., Dewez, D., Ikoma, D., Grossman, A.R., Kottke, T. and Mittag, M. (2012), A flavin-binding cryptochrome photoreceptor responds to both blue and red light in *Chlamydomonas reinhardtii* [published online ahead of print July 6, 2012]. *The Plant Cell*, doi: http://dx.doi.org/10.1105/tpc.112.098947. Accessed July 10, 2012.

Bruce, V. (1970), The biological clock in *Chlamydomonas reinhardi*. *Journal of Eukaryotic Microbiology*, 17, 328–334.

Bruce, V. (1972). Mutants of the biological clock in *Chlamydomonas reinhardi*. *Genetics*, 70(4), 537-548.

Cashmore, A. (2003). Cryptochromes: enabling plants and animals to determine circadian time. *Cell*, 114(5), 537-543.

Coesel, S., Mangogna, M., Ishikawa, T., Heijde, M., Rogato, A., Finazzi, G., Todo, T., Bowler, C. and Falciatore, A. (2009). Diatom PtCPF1 is a new cryptochrome/photolyase family member with DNA repair and transcription regulation activity. *EMBO Reports*, 10(6), 655-661.

Deininger, W., Kröger, P., Hegemann, U., Lottspeich, F. and Hegemann, P. (1995). Chlamyrhodopsin represents a new type of sensory photoreceptor. *The EMBO Journal*, 14(23), 5849-5858.

Devlin, P. (2002). Signs of the time: environmental input to the circadian clock. *Journal of Experimental Botany*, 53(374), 1535-1550.

Devlin, P. and Kay, S. (2001). Circadian photoreception. *Annual Review of Physiology*, 63, 677-694.

Djouani-Tahri, el-B., Christie, J.M., Sanchez-Ferandin, S., Sanchez, F., Bouget, F.Y. and Corellou, F. (2011). A eukaryotic LOV-histidine kinase with circadian clock function in the picoalga *Ostreococcus*. *The Plant Journal*, 65(4), 578-588.

Emery, P., So, W., Kaneko, M., Hall, J. and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*, 95(5), 669-679.

Feldman, J. and Hoyle, M. (1973). Isolation of circadian clock mutants of *Neurospora crassa*. *Genetics*, 75(4), 605-613.

Forbes-Stovall, J. (2011). Action spectrum for photoentrainment of the circadian clock in wild-type *Chlamydomonas reinhardtii*. M.S. Thesis, Western Kentucky University, Bowling Green, KY.

Frank, K.D. and Zimmerman, W.F. (1969). Action spectra for phase shifts of a circadian rhythm in *Drosophila*. *Science*, 163(3868), 688-689.

Fuhrmann, M., Stahlberg, A., Govorunova, E., Rank, S. and Hegemann, P. (2001). The abundant retinal protein of the *Chlamydomonas* eye is not the photoreceptor for phototaxis and photophobic responses. *Journal of Cell Science*, 114(Pt 21), 3857-63.

Gaskill, C. (2009). Towards an action spectrum for photoentrainment of the *Chlamydomonas reinhardtii* circadian clock. M.S. Thesis, Western Kentucky University, Bowling Green, KY.

Gaskill, C., Forbes-Stovall, J., Kessler, B., Young, M., Rinehart, C. and Jacobshagen S. (2010). Improved automated monitoring and new analysis algorithm for circadian phototaxis rhythms in *Chlamydomonas*. *Plant Physiology and Biochemistry*, 48(4), 239-246.

Gonzalez-Ballester, D., Pootakham, W., Mus, F., Yang, W., Catalanotti, C., Magneschi, L., de Montaigu, A., Higuera, J.J., Prior, M., Galván, A., Fernandez, E. and Grossman, A.R. (2011). Reverse genetics in *Chlamydomonas*: a platform for isolating insertional mutants. *Plant Methods*, 7:24. doi: 10.1186/1746-4811-7-24 Accessed on July 10, 2012.

Goto, K. and Johnson, C. (1995). Is the cell division cycle gated by a circadian clock? The case of *Chlamydomonas reinhardtii*. *The Journal of Cell Biology*, 129(4), 1061-1069.

Harmer, S., Hogenesch, J., Straume, M., Chang, H., Han, B., Zhu, T., Wang, X., Kreps, J. and Kay, S. (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science*, 290(5499), 2110-2113.

Harris E, (1989) The *Chlamydomonas* Sourcebook: a comprehensive guide to biology and laboratory use. Academic Press, San Diego.

Harris, E. (2001). *Chlamydomonas* as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology*, 52, 363-406.

Hattar, S., Liao, H., Takao, M., Berson, D. and Yau, K. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science*, 295(5557), 1065-1070.

Heijde, M., Zabulon, G., Corellou, F., Ishikawa, T., Brazard, J., Usman, A., Sanchez, F., Plaza, P., Martin, M., Falciatore, A., Todo, T., Bouget, F.Y. and Bowler, C. (2010). Characterization of two members of the cryptochrome/photolyase family from *Ostreococcus tauri* provides insights into the origin and evolution of cryptochromes. *Plant, Cell & Environment*, 33(10), 1614-1626.

Heintzen, C., Loros, J.J. and Dunlap, J.C. (2001). The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting. *Cell*, 104(3), 453-464.

Huang, K., Merkle, T. and Beck, C.F. (2002). Isolation and characterization of a *Chlamydomonas* gene that encodes a putative blue light photoreceptor of the phototropin family. *Physiologia Plantarum*, 115(4), 613-622.

Huang, K. and Beck, C. (2003). Phototropin is the blue light receptor that controls multiple steps in the sexual life cycle of the green alga *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, 100(10), 6269-6274.

Huang, K., Kunkel, T. and Beck, C. (2004). Localization of the blue light receptor phototropin to the flagella of the green alga *Chlamydomonas reinhardtii*. *Molecular Biology of the Cell*, 15(8), 3605-3614.

Johnson, C.H., Kondo, T. and Hastings, J.W. (1991). Action Spectrum for Resetting the Circadian Phototaxis Rhythm in the CW15 Strain of *Chlamydomonas*: II. Illuminated Cells. *Plant Physiology*, 97(3), 1122-1129.

Johnson, C.H., Elliott, J.A. and Foster, R. (2003). Entrainment of circadian programs. *Chronobiology International*, 20(5), 741-774.

Kateriya, S., Nagel, G., Bamberg, E. and Hegemann, P. (2004). "Vision" in single-celled algae. *News in Physiological Sciences*, 19, 133-137.

Kim, E.J. and Cerutti, H. (2009). Targeted gene silencing by RNA interference in *Chlamydomonas*. *Methods in Cell Biology*, 93, 99-110.

Kondo, T., Johnson, C. and Hastings, J. (1991). Action Spectrum for Resetting the Circadian Phototaxis Rhythm in the CW15 Strain of *Chlamydomonas*: I. Cells in Darkness. *Plant Physiology*, 95(1), 197-205.

Konopka, R. and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 68(9), 2112-2116.

Krishnan, B., Levine, J., Lynch, M., Dowse, H., Funes, P., Hall, J., Hardin, P. and Dryer, S. (2001). A new role for cryptochrome in a *Drosophila* circadian oscillator. *Nature*, 411(6835), 313-317

Kucho, K., Okamoto, K., Tabata, S., Fukuzawa, H. and Ishiura, M. (2005). Identification of novel clock-controlled genes by cDNA macroarray analysis in *Chlamydomonas reinhardtii*. *Plant Molecular Biology*, 57(6), 889-906.

Kume, K., Zylka, M., Sriram, S., Shearman, L., Weaver, D., Jin, X., Maywood, E., Hastings, M. and Reppert S. (1999). mCRY1 and mCRY2 are essential

components of the negative limb of the circadian clock feedback loop. *Cell*, 98(2), 193-205.

Mergenhagen, D. and Mergenhagen, E. (1987). The biological clock of *Chlamydomonas reinhardii* in space. *European Journal of Cell Biology*.

Millar, A.J., Straume, M., Chory, J., Chua, N.H. and Kay, S.A. (1995). The regulation of circadian period by phototransduction pathways in *Arabidopsis*. *Science*, 267(5201), 1163-1166.

Mittag, M., Kiaulehn, S. and Johnson C. (2005). The circadian clock in *Chlamydomonas reinhardtii*. What is it for? What is it similar to? *Plant Physiology*, 137(2), 399-409.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D. and Baulcombe, D. (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *The Plant Journal*, 58(1), 165-174.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P. and Bamberg, E. (2003). *Proceedings of the National Academy of Sciences of the United States of America*, 100(24), 13940-13945.

Neuhoff, V., Arold, N., Taube, D. and Ehrhardt W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, 9(6), 255-262.

Pittendrigh, C. (1993). Temporal organization: reflections of a Darwinian clock-watcher. *Annual Review of Physiology*, 55, 16-54.

Quail, P.H. (1991). Phytochrome: a light-activated molecular switch that regulates plant gene expression. *Annual Review of Genetics*, 25, 389-409.

Reisdorph, N.A. and Small, G.D. (2004). The CPH1 gene of *Chlamydomonas reinhardtii* encodes two forms of cryptochrome whose levels are controlled by light-induced proteolysis. *Plant Physiology*, 134(4), 1546-1554.

Rochaix, J. (2001). Assembly, function, and dynamics of the photosynthetic machinery in *Chlamydomonas reinhardtii*. *Plant Physiology*, 127(4), 1394-1398.

Rohr, J., Sarkar, N., Balenger, S., Jeong, B.R. and Cerutti, H. (2004). Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*. *The Plant Journal*, 40(4), 611-621.

Schroda, M. (2006). RNA silencing in *Chlamydomonas*: mechanisms and tools. *Current Genetics*, 49(2), 69-84.

Silflow, C. and Lefebvre, P. (2001). Assembly and motility of eukaryotic cilia and flagella. Lessons from *Chlamydomonas reinhardtii*. *Plant Physiology*, 127(4), 1500-1507.

Sineshchekov, O., Jung, K. and Spudich, J. (2002). Two rhodopsins mediate phototaxis to low- and high-intensity light in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America*, 99(13), 8689-8694.

Sjoblad, R. and Frederikse, P. (1981). Chemotactic responses of *Chlamydomonas reinhardtii*. *Molecular and Cellular Biology*, 1(12), 1057-1060.

Small, G., Min, B. and Lefebvre, P. (1995). Characterization of a *Chlamydomonas reinhardtii* gene encoding a protein of the DNA photolyase/blue light photoreceptor family. *Plant Molecular Biology*, 28(3), 443-454.

Somers, D., Devlin, P. and Kay, S. (1998). Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science*, 282(5395), 1488-1490.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M. and Hall, J.C. (1998). The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell*, 95(5), 681-692.

Taiz, L. and Zeiger, E. *Plant physiology*. 4th ed. Sinauer Associates Inc, 2006.

van der Horst, G., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. and Yasui, A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*, 398(6728), 627-630.

Wang, H., Ma, L., Li, J., Zhao, H. and Deng, X. (2001). Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. *Science*, 294(5540), 154-158.

Werner, R. (2002). *Chlamydomonas reinhardtii* as a unicellular model for circadian rhythm analysis. *Chronobiology International*, 19(2), 325-343.

Yamasaki, T., Miyasaka, H. and Ohama, T. (2008). Unstable RNAi effects through epigenetic silencing of an inverted repeat transgene in *Chlamydomonas reinhardtii*. *Genetics*, 180(4), 1927-1944.

Zhao, T., Wang, W., Bai, X. and Qi, Y. (2009). Gene silencing by artificial microRNAs in *Chlamydomonas*. *The Plant Journal*, 58(1), 157-164.