Published in final edited form as:
Plant Physiol Biochem. 2014 February ; 75: 14–23. doi:10.1016/j.plaphy.2013.12.002.

Chlamydomonas reinhardtii strain CC-124 is highly sensitive to blue light in addition to green and red light in resetting its circadian clock, with the blue-light photoreceptor plant cryptochrome likely acting as negative modulator

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Abstract

The unicellular green alga *Chlamydomonas reinhardtii* has long served as model organism for studies on the circadian clock. This clock is present in all eukaryotes and some prokaryotes allowing them to anticipate and take advantage of the daily oscillations in the environment. Although much is known about the circadian clock in *C. reinhardtii*, the photoreceptors mediating entrainment of the clock to the daily changes of light remain obscure. Based on its circadian rhythm of phototaxis as a reporter of the clock's phase, we show here that *C. reinhardtii* strain CC-124 is highly sensitive to blue light of 440 nm when resetting its circadian clock upon light pulses. Thus, CC-124 differs in this respect from what was previously reported for a cell wall-deficient strain. An action spectrum analysis revealed that CC-124 also responds with high sensitivity to green (540 nm), red (640–660 nm), and possibly UV-A (≤400 nm) light, and therefore shows similarities as well to what has been reported for the cell wall-deficient strain. We also investigated two RNA interference strains with reductions in the level of the blue light photoreceptor plant cryptochrome (CPH1). One of them, the strain with the greater reduction, surprisingly showed an increased sensitivity in clock resetting upon blue light pulses of 440 nm. This increase in sensitivity reverted to wild-type levels when the RNA interference strain reverted to wild-type protein levels. It suggests that plant cryptochrome in *C. reinhardtii* could function as negative rather than positive modulator of circadian clock resetting.

Keywords

Circadian clock; resetting; cryptochrome; *Chlamydomonas*; CC-124; blue light; entrainment

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1. Introduction

The circadian clock is a biochemically based endogenous timer of nearly all eukaryotes and some prokaryotes. The clock synchronizes many metabolic, physiological, and behavioral processes with the daily environmental cycles of light and temperature. The processes retain their approximately 24-hour rhythms even in the absence of environmental time cues. The self-sustaining rhythmicity of the circadian clock allows an organism to anticipate the daily changes so that it may both overcome and take advantage of the periodic nature of the environment. For example, photosynthetic carbon fixation is substantially increased in plants with proper circadian clocks [1]. Since it is necessary for the clock to remain in synchrony with the daily environmental oscillations, it can be entrained based upon environmental time cues such as the daily light/dark changes.

Chlamydomonas reinhardtii is a unicellular eukaryotic green alga that has long served as model organism for a variety of cellular processes [2], among them the circadian clock [3,4]. It shows many well-characterized circadian rhythms of behavior, physiology, and gene expression [5–7]. The measurement of its rhythm of phototaxis (swimming towards or away from light) in the form of an accumulation rhythm has been automated [8,9], as has the measurement of a rhythm of gene expression that is based upon reporting by firefly luciferase [10]. We recently built our own machine for automated phototaxis rhythm measurements based on the previously employed principle but with some optimizations [11].

Although much is already known about the circadian clock in *C. reinhardtii* [3,4] including some of the signal transduction mechanisms involved in its resetting [12], the photoreceptors that mediate entrainment to the daily light/dark cycles remain obscure. A promising candidate for this function is the alga's recently described plant cryptochrome (CPH1) [13]. The role of plant cryptochrome in *C. reinhardtii* so far is unknown, but an involvement in circadian clock resetting has been suggested, since this is also a function of plant cryptochromes in higher plants [14]. The role that plant cryptochrome plays in *C. reinhardtii* has become even more interesting with the recent discovery of an additional cryptochrome of the animal type in the alga's genome [15]. This animal cryptochrome was found to mediate light-regulated expression of several genes [16]. How these two cryptochromes in *C. reinhardtii* might or might not overlap in their function and whether one of them or perhaps even both mediate light-induced resetting of the circadian clock are now particularly intriguing questions.

There is one finding nonetheless that would point to a different main photoreceptor for circadian clock entrainment in *C. reinhardtii* than either plant or animal cryptochrome. An action spectrum analysis determined that dark-adapted cells are highly sensitive to green and red light in their resetting response but only little sensitive to blue light [9]. Although animal cryptochrome in *C. reinhardtii* can also respond to light of wavelengths other than blue, it as well as plant cryptochrome are both activated by blue light [16,17]. Since the action spectrum experiments were conducted with a cell wall-deficient strain and under conditions that we were able to improve on when using strain CC-124 [11], we tested the resetting response of CC-124 upon blue light pulses.

We found that under our conditions *C. reinhardtii* CC-124 is well able to respond to 15 min low intensity pulses of 440 nm blue light with resetting of its circadian clock. When determining the entire action spectrum between the wavelengths of 400 and 700 nm at 20 nm intervals, we further found that the strain also responds to green (540 nm) and red (640–660 nm) light with a sensitivity nearly as high as that for blue light. We additionally investigated whether plant cryptochrome is involved in the blue-light response by making

use of the arginine-requiring mutant CC-48 to create two RNA interference (RNAi) strains. We found that only the RNAi strain with the stronger reduction showed a significant difference to the parent strain in its resetting response to blue light. Surprisingly, the reduction of plant cryptochrome caused a higher sensitivity to blue light, which was lost after the strain had reverted to wild-type levels of the protein. This suggests that in *C. reinhardtii* CC-48 plant cryptochrome has an inhibitory rather than an activating function in circadian clock resetting by blue light.

2. Results

2.1 Blue light resets the circadian clock of *C. reinhardtii* CC-124 with high efficacy

When *C. reinhardtii* strain CC-124 is given a 15 min blue light pulse of 440 nm at about the middle of the night (7 h into the dark phase of a 12 h light/12 h dark cycle), it responds by resetting its circadian clock as made evident by the shift in phase of its circadian phototaxis rhythm (Figure 1). It will delay the rhythm, since the peaks and troughs occur later as compared to control cultures that did not receive a light pulse. The extent of delay depends on the intensity of the blue light pulse with a maximum shift of between 3 and 4 CT units. A CT (circadian time) unit is equal to 1/24th of the period of the free-running rhythm and therefore about 1 hour. The shift is specific to the blue light pulse and not caused by the handling of the cultures or by some possible light contamination of the room, because mock control cultures that are handled identically except that the shutter of the light pulse device is not opened during the 15 min light pulse do not show this phase shift (Figure 1). Analysis of variance confirms that there is no significant difference in phase shift between the mock control cultures that were placed in the different slots of the light pulse device, but there is a significant difference in phase shift between cultures exposed to different intensities of 440 nm light (α=0.05). Our results suggest that CC-124 is highly sensitive to pulses of 440 nm blue light in its circadian clock resetting response. This is different from what was reported for the cell wall-deficient strain CW15 [9], which is not particularly sensitive to blue light but rather responds to green and red light with high efficacy.

2.2 Green and red light also reset the circadian clock efficiently in *C. reinhardtii* CC-124

Since strain CC-124 was so remarkably sensitive to 440 nm blue light in its circadian resetting response, we decided to also test other wavelengths for similarly high sensitivities. We tested light pulses of wavelengths between 400 and 700 nm at 20 nm intervals, altogether 16 different wavelengths. As shown in Figure 2, the light intensity response curves for phase shifting of the circadian phototaxis rhythm vary between different wavelengths. For some wavelength, like 440 nm, 540 nm, 640 nm and 660 nm, the curves occur further to the left in the graph towards lower light intensities indicating that *C. reinhardtii* CC-124 has a greater sensitivity for light at these wavelengths, while for other wavelengths, like 460 nm, 500 nm, and 680 nm, the curves are further to the right towards higher light intensities and therefore indicate a greatly decreased sensitivity. We did not observe any shift in phase for light at 700 nm wavelength even at the highest intensity tested. The shapes of the curves are more or less similar with similar slopes at around the half-maximal phase shift and also with similar extent of phase shift at light saturation, at least for those wavelengths where saturation was reached. A notable exception to the overall shape is the curve for 420 nm, which shows a slope that is considerably shallower.

In order to quantify the sensitivity of *C. reinhardtii* CC-124 to light of the various wavelengths, we created a model for each wavelength response by determining the best-fitting curve according to the equation $y=c/(1+b*e^{-kx})$, where *c* is the maximal phase shift at saturating light intensity. Since for several wavelengths the light intensities we used were not high enough to cause saturation and since the slopes were roughly the same in

nearly all cases, we set *c* to −4 CT units in the equation. As shown in Figure 3 for the three wavelengths that were most effective, the models follow the actual data quite closely with R^2 values of 0.963, 0.982, and 0.983 for 440, 540, and 660 nm, respectively. Figure 3 also shows the model for 420 nm, where the slope of the actual data curve was shallower, which is reflected in the model. The R^2 value for this model is 0.961.

Based on the model we created for each of the 16 wavelengths, we calculated the light intensity required to shift the phase by −2 CT units or half of the maximal shift. The reciprocal of this value is a relative measure for sensitivity with a higher number indicating a higher sensitivity for the particular wavelength. Therefore, in the action spectrum obtained from these data, the peaks indicate spectral regions of high sensitivity. As shown in Figure 4, the action spectrum for circadian clock resetting of *C. reinhardtii* CC-124 shows four regions of high sensitivity: in the red with a peak between 640 and 660 nm, in the green with a peak at 540 nm, in the blue with a peak at 440 nm, and probably in the UV-A region as indicated by the marked increase in sensitivity from 420 to 400 nm wavelength. The difference in efficacy between these regions is only slight, with 440 nm the strongest, with 540 nm at about 75% of 440 nm, 400 nm about 50%, and 660 nm about 40%. Interestingly, the previously published action spectrum for the cell wall-deficient strain CW15 [9] also shows high sensitivity in the red (peak at 660 nm) and in the green (peak at 520 to 540 nm) as well as a marked increase in sensitivity from 420 to 400 nm. It means that the strong response to 440 nm blue light is the only significant difference that distinguishes our experimental outcomes with CC-124 from those reported for CW15.

2.3 Plant cryptochrome likely represses circadian clock resetting by blue light in *C. reinhardtii*

To test our hypothesis that the previously described photoreceptor plant cryptochrome in *C. reinhardtii* mediates blue light-induced circadian clock resetting, we isolated two RNA interference strains, cryRNAi#16 and cryRNAi#18, with about 75% and 50% reduction, respectively (Figure 5). The RNAi strains are derived from the arginine-requiring mutant CC-48, which was chosen because of the ARG7 gene in our construct that served as *C. reinhardtii* marker by restoring the ability of transformants to grow without exogenous arginine and because CC-48 shows a robust circadian rhythm of phototaxis under our conditions. When testing the ability of the two RNAi strains to shift the phase of their phototaxis rhythm upon a 440 nm blue light pulse, we were surprised to discover that none showed a decrease in this ability. On the contrary, one of the two strains, the one with the larger cryptochrome reduction, showed a strong increase in sensitivity towards blue light compared to the parent strain CC-48. As shown in Figure 5, the light intensity curve of strain cryRNAi#16 for phase shifting of the circadian phototaxis rhythm lies further to the left of the curve for the parent strain and therefore towards lower light intensities. The curve for strain cryRNAi#18 on the other hand is much closer to that of the parent strain. Analysis of variance revealed that the cryRNAi#16 curve is significantly different from the curve of the parent strain (α=0.05), which is not the case for the cryRNAi#18 curve.

There is the possibility that the increased sensitivity of strain cryRNAi#16 to blue light for circadian clock resetting is due to an unintended side effect of the RNAi technique rather than directly the effect of the plant cryptochrome knock-down. The double-stranded RNA expressed from our RNAi construct could have induced off-target effects by also silencing another gene with some sequence similarity to plant cryptochrome [18]. Alternatively, transformation with the RNAi construct could have resulted in a mutation due to the integration of the transgene into the genome or due to some genome deletion. We were able to investigate the latter possibilities, because cryRNAi#16 reverted to wild-type levels of plant cryptochrome over time (Figure 6). Reversions of RNAi-induced knock-downs have

commonly been reported for *C. reinhardtii* and are usually due to its ability to silence transgenes [19]. As Figure 6 demonstrates, the loss of plant cryptochrome knock-down is correlated with a decrease in blue-light sensitivity of circadian clock resetting to about the level of the parent strain. It suggests that the previously observed increased sensitivity was most likely due to the low levels of plant cryptochrome protein and that therefore plant cryptochrome is most likely involved in blue light-induced circadian clock resetting in *C. reinhardtii*, however, with inhibiting rather than activating function.

3. Discussion

3.1 Wavelengths of light effective in resetting the circadian clock of *C. reinhardtii* CC-124

The experiments described here demonstrate that *C. reinhardtii* strain CC-124 is highly sensitive to 440 nm blue light in its ability to reset the circadian clock upon light pulses (Figure 1). We also demonstrate that mock control cultures do not show this resetting, although they were manipulated in exactly the same way except that the shutter of our light pulse device was kept closed. It shows that resetting is entirely due to the blue light pulse and not to some other experimental condition.

Our results are in contrast to previous reports for the cell wall-deficient strain CW15 [9], which did not show high sensitivity to blue light for circadian clock resetting in dark-adapted cells. Only when the cells were adapted to constant dim light [20] was a blue-light dominated resetting response observed. Under these conditions, resetting was shown to be mediated by photosynthesis and required pulses of light with intensities about 1000 times higher and with durations 24 times longer (6 h versus 15 min) than the effective light pulses for dark-adapted cells.

Interestingly, the wavelengths of light that did show high efficacy in resetting of the circadian clock of dark-adapted CW15 [9] are also particularly effective in strain CC-124 (Figure 4), i.e. green (520–540 nm), red (640–660 nm) and potentially UV-A (≤400 nm). It brings up the question of what might cause the difference between the strains in their blue-light response. Generally, the difference could be due either to some particular difference between the strains or to differences in experimental conditions.

Both strains differ in their genetic background, although they also show similarities. For example, although CC-124 is often called one of the “wild-type” strains, it actually carries a mutation in the *nit1* and *nit 2* gene, which renders it unable to grow on nitrate as nitrogen source [2]. These two mutations are also common in many other laboratory strains of *C. reinhardtii*, including CW15 [2]. One difference in the genetic background between the strains is their mating type locus (mt^- for CC-124, mt^+ for CW15). Another difference is that CW15 carries a not entirely characterized mutation that renders it cell wall-deficient [2], whereas CC-124 has a wild-type cell wall. A third difference is that strain CC-124 carries the $agg1^-$ allele [21], a “modified phototactic aggregation” allele thought to represent a naturally occurring genetic polymorphism, whereas CW15 carries the $agg1^+$ allele. The $agg1^-$ allele causes CC-124 to show negative phototaxis (to swim away from the light source) under all environmental conditions investigated so far, including conditions when strains with the $agg1^+$ allele will show positive phototaxis (swim towards the light source). Notably, as we have demonstrated before [11] and confirmed in this report, our CC-124 strain shows an accumulation pattern in the phototaxis machine that is similar to the pattern reported for $agg1^+$ strains in that it shows all characteristics of a true circadian rhythm with continued rhythmicity under free-running conditions and resetting to environmental time cues such as light pulses. The phase of the rhythm is also similar with maximum accumulation during the middle of the subjective day (Table 1). The molecular basis for agg1 is not known other than that it is localized on linkage group XIV [22]. Interestingly,

CC-124 was recently reported to show positive phototaxis after treatment with reactive oxygen species, which are thought to represent signaling intermediates that allow the sign of phototaxis to be regulated by the rate of photosynthesis [23].

Based on these genetic differences between CW15 and CC-124, one possible reason for their different sensitivities to blue light when resetting their circadian clock could be the difference in their cell walls. Proper resetting might be dependent on a proper wild-type cell wall as is found in CC-124. The requirement for a wild-type cell wall might be based on required interaction between the photoreceptor and the cell wall, possibly due to localization of the photoreceptor in the plasma membrane. Another potential reason for the different resetting response that is based on their genetic differences is the different agg1 allele they contain. It is possible that the $agg1^{-}$ allele in CC-124 confers a greater sensitivity to blue light that leads to both negative phototaxis and circadian clock resetting at already lower light intensities whereas the $agg1^{+}$ allele in CW15 makes the strain require higher intensities for these two responses. As a third possibility, a yet undiscovered genetic difference could be the reason for the different blue-light resetting responses of the strains.

Differences in experimental design that was used with each strain are another possible reason for the different sensitivities to blue light. Resetting of the circadian clock for cell wall-deficient strain CW15 was also monitored via its rhythm of phototaxis as we have done here for CC-124, but for CW15 white background light was used between phototaxis measurements [9]. Background light was needed for CW15 to show a rhythm of phototaxis that was persistent. As shown in the study, the act of placing the cultures into the phototaxis machine by itself could already lead to a phase shift [9]. Resetting of the circadian clock was therefore potentially due to the combined effect of the light pulse applied before the rhythm measurements and the effect of placing the cells from their dark environment into the phototaxis machine with its white background light some time after the pulse [9]. We did not use white background light between phototaxis measurements and we previously showed that placement of our cultures into the phototaxis machine under our conditions does not cause significant phase shifts [11]. Interestingly, because of the combination of two potential phase shifting events for CW15, another possible reason for the low sensitivity of this strain to blue light-induced resetting could be that in this particular case phase shifting due to the light pulse and due to the placement of the cultures into the phototaxis machine canceled each other out, but that in the case of green and red light no cancellation occurred.

Generally, the circadian resetting response to blue light by CC-124 does not seem to exhibit a simple pattern. On the one hand, the action spectrum in this region (400–500 nm) does not show the broad “three-finger” curve usually found for responses mediated either by photosynthesis or by the two types of blue light photoreceptors reported for *C. reinhardtii*, i.e. phototropins and cryptochromes. The curve shows a narrower pattern instead that centers around the 440 nm wavelength (Figure 4). On the other hand, the steepness of the light intensity response curve varies for different blue light wavelengths (Figure 2). In particular, the intensity response curve for light of 420 nm is much shallower. It suggests that the response at this wavelength could be due to the interaction of two or more photoreceptors and therefore that the response to blue light might be unexpectedly complex.

3.2 Plant cryptochrome as a possible negative modulator of blue light-induced clock resetting

When testing for the involvement of the blue light photoreceptor plant cryptochrome in circadian clock resetting of *C. reinhardtii* upon blue light pulses, we were surprised to find that this cryptochrome seemed to inhibit rather than promote resetting. Of the two RNAi strains with reduced plant cryptochrome levels, the one with the greater reduction was significantly more sensitive to 440 nm blue light than the parent strain (Figure 5).

RNAi is a powerful technique for reverse genetics studies, but it can also lead to results that turn out to be artifacts. One problem is that transformation with the RNAi construct can lead to mutations. Therefore, the observed effect can be the result of a secondary mutation rather than the specific knock-down. This is particular the case for *C. reinhardtii*, since integrations of transgenes into its genome overwhelmingly occur through random integrations rather than homologous recombinations [24]. Consequently, mutations may arise due to the specific site of integration or due to additional deletions of genomic DNA during the integration process. We were able to test this particular possibility, since our RNAi strain reverted to wild-type levels of plant cryptochrome over time (Figure 6). Reversions of RNAi strains is a phenomenon commonly reported for *C. reinhardtii* and is usually due to the alga's ability to silence transgenes [19]. Because of the possibility for reversion, we collected a sample for the analysis of plant cryptochrome levels every time our RNAi cultures received a light pulse, taking the sample directly from an aliquot culture at the time of the pulse (see Materials and Methods section). If RNAi reversion occurs due to transgene silencing, the genome's DNA sequence including any possible mutation is not changed, only the rate of transgene expression. Therefore, because our reverted RNAi strain also showed reversion of its increased sensitivity to blue light (Figure 6), it suggests that the original sensitivity to blue light was not due to an additional unintended mutation in the RNAi strain.

The other possible problem with the RNAi technique is that it might cause off-target effects [18]. The small interfering RNAs produced from the double-stranded RNA in the organism might knock down other genes as well due to some sequence identities. We cannot exclude off-target effects here. To do that, additional RNAi strains with various double-stranded RNAs that represent other regions of the plant cryptochrome gene would be needed. Alternatively, use of the recently developed artificial microRNA techniques for *C. reinhardtii* [25,26] would also lower the potential for off-target effects.

One interesting aspect that we were able to determine, however, is whether the RNAi strains differ from the parent strain in the parameters of their circadian phototaxis rhythm. As demonstrated in Table 1, there were no major differences between the rhythm of the parent strain CC-48 and its derived RNAi strains in either period or phase for the control cultures that did not receive a phase shifting light pulse. It suggests that the reduction in plant cryptochrome or generally our RNAi manipulations had no effect on how phototaxis is regulated by the circadian clock under our free-running conditions.

In conclusion, our data suggest that plant cryptochrome is likely to function as negative modulator of circadian clock resetting upon blue light pulses in *C. reinhardtii* CC-48, although it is not entirely proven yet.

The strain of *C. reinhardtii* that we used for our plant cryptochrome studies (CC-48) was not the same as the one we used for our action spectrum studies (CC-124) due to the *Chlamydomonas* marker that was part of our RNAi construct. Interestingly, a comparison of the two strains in their circadian resetting response to 440 nm blue light reveals that CC-48 is much less sensitive to blue light than CC-124. When analyzing the response by CC-48 in the same way as for CC-124, the intensity needed for a half maximal shift is nearly 50 fold higher in the case of CC-48 as compared to CC-124. However, whether this is due to a lower sensitivity to blue light in particular or to light in general is not clear. Since CC-48 has a wild-type cell wall like CC-124 but presumably the $agg1^{+}$ allele, it is tempting to speculate that the $agg1^{-}$ allele of CC-124 might not only confer its higher blue light sensitivity, but it might even accomplish this through a weakened inhibitory function of plant cryptochrome. Although the agg1 gene cannot be identical with the plant cryptochrome gene itself, since

plant cryptochrome is located on chromosome 6 but agg1 on chromosome 13 (linkage group XIV), agg1 could encode a signaling intermediate in plant cryptochrome function.

A photoreceptor acting as negative modulator of circadian clock resetting has been described before with the example of the blue light photoreceptor VIVID in *Neurospora* [27]. In this fungus, the WHITE COLLAR COMPLEX (WCC) acts as positive element in the central oscillator's transcription/translation feedback loop based on its transcriptional activator function. WCC also acts as blue light photoreceptor for circadian clock resetting and many other blue light-induced processes through its WHITE COLLAR-1 (WC-1) subunit [28]. VIVID, when activated by blue light, will inhibit WCC by competing for binding of the WCC's two subunits and thus by forming alternative heterodimers [29]. Because the gene encoding VIVID is light-inducible, high levels of VIVID are only formed after some time, thereby delaying its repressive action upon light [30]. This delay is the basis for photoadaptation in *Neurospora* causing light-inducible processes to be transient despite continuing illumination and allowing the organism to respond to further increases in light intensity. Since expression of the gene encoding VIVID shows a circadian rhythm and since its light-inducibility is gated by the circadian clock [27,30], VIVID causes *Neurospora* to be particularly sensitive to light at dawn and prevents resetting of the circadian clock by moonlight [29].

The biology of plant cryptochrome of *C. reinhardtii* is not yet well understood, but what is known demonstrates some interesting differences to VIVID of *Neurospora*. Under 12 h light/12 h dark cycle condition, protein levels of plant cryptochrome oscillate with the highest amounts towards the end of the dark period [13], while VIVID shows its highest amount during the early light period [30]. The oscillating pattern of plant cryptochrome is at least partially due to its rapid degradation in the light [13] whereas that of VIVID to the light induction of its gene [30]. This difference compared to VIVID suggests that plant cryptochrome can be expected to aid the organism somewhat differently in its response to the environment.

In general, the function of plant cryptochrome in *C. reinhardtii* might be to allow the alga to fine-tune its response according to the time-of-day and to the spectral quantity and quality of the incident light. This might also apply to other light-regulated processes aside from the resetting of the circadian clock, as has been reported for higher plants, where this type of photoreceptor additionally regulates activities like hypocotyl elongation, flowering time, anthocyanin production, and stomatal opening [31].

3.3 Other photoreceptors possibly involved in circadian clock resetting of *C. reinhardtii*

The results reported here demonstrate that *C. reinhardtii* strain CC-124 is particularly sensitive to blue (440 nm), green (540 nm) and red (640–660 nm) light, and probably to UV-A light (≤400 nm), when resetting its circadian clock (Figure 4). These sensitivities could be mediated by some of the known photoreceptors in *C. reinhardtii*, by some yet undiscovered photoreceptors or by photosynthesis. Although we did not directly test this, we think it unlikely that photosynthesis is mediating the responses to blue and red light for three reasons: (1) the light intensities required are very low (1.5×10^{-9} and 3.9×10^{-9} mol photons m^{-2} s^{-1} for 15 min for 440 nm and 660 nm, respectively, to achieve 50% maximal shift), (2) the curve of the action spectrum differs from that for photosynthesis (the blue region in particular would otherwise be much broader), and (3) the red light response of dark-adapted CW15 was shown to be unaffected by a photosynthesis inhibitor [9].

The known photoreceptors in *C. reinhardtii* belong to the phototropin, cryptochrome and rhodopsin types [32]. There are at least six genes in the *C. reinhardtii* genome encoding rhodopsins [32], two of which were shown to mediate phototaxis [33]. These two are

unlikely candidates for clock resetting, since they were reported to induce maximal action at about 470 and 507 nm. However, one or more of the other yet uncharacterized rhodopsins could be responsible for clock resetting upon pulses of green (540 nm) light. Interestingly, a recent spectroscopic study of one of those rhodopsins found that the dark form absorbs UV-A with a maximum of 380 nm, turning it into a stable form that can be switched back into the dark form by blue light [34]. It shows that rhodopsins might even be candidates for the UV-A and blue light responses.

The other two classes of photoreceptors, the phototropins and cryptochromes, have generally been considered blue light receptors, although this might be too narrow a view for some of the cryptochromes. There is a single phototropin encoded in *C. reinhardtii* that was shown to mediate several blue-light effects, like the ones regulating the sexual life cycle [35] and the expression of some genes involved in chlorophyll and carotenoid biosynthesis [36]. The phototropins of higher plants are most likely not involved in circadian clock resetting, since phototropin mutants did not show observable circadian defects. Also, absorption/action spectra of phototropins generally show a characteristic broad “three-finger” pattern in the 400–500 nm region, which is not the case for the action spectrum presented here.

There is a second cryptochrome in addition to the plant cryptochrome encoded in the *C. reinhardtii* genome, which was shown to also act as photoreceptor [16]. The sequence of this cryptochrome is most closely related to animal cryptochromes [15]. Recent analysis of this animal cryptochrome in *C. reinhardtii* surprisingly revealed an absorption and action pattern quite different from that of plant cryptochromes [16]. In addition to the typical broad and “three-fingered” pattern in the blue, the animal cryptochrome of *C. reinhardtii* also showed an absorption maximum and a physiological response in the red (633 nm) and yellow (585 nm). The physical basis of this difference was reported to lie in the particular oxidation state of the chromophore in the dark form of the photoreceptor. Whereas FAD in plant cryptochrome of *C. reinhardtii* occurs in its fully oxidized state [18], FAD of animal cryptochrome occurs in its neutral radical state [16].

Animal cryptochrome in *C. reinhardtii* was reported to mediate light induction of a number of genes, among them some that are involved in the circadian clock [16]. Whether the photoreceptor also mediates resetting of the circadian clock remains to be determined. It could be responsible for the blue and red light effects, although it is then unclear why no significant effect was observed in the yellow.

In summary, our data suggest that entrainment of the *C. reinhardtii* circadian clock to the daily oscillations of light in the environment is quite complex as must perhaps be expected of an organism, for which light is of the utmost importance for survival, but more research into this interesting area of perception is needed.

4. Materials and Methods

4.1 Strains and growth conditions

Chlamydomonas reinhardtii strain CC-124 (137c mt^-) was obtained from Christoph Beck (Albert-Ludwig University, Freiburg, Germany) and strain CC-48 (arg2, mt^+) from the Chlamydomonas Center culture collection (chlamycollection.org). Since strain CC-48 is auxotrophic for arginine, all culture media for this strain also contained L-arginine at a concentration of 0.2 mg/ml. For all light pulse experiments, cells were grown as described [11]. Briefly, 10^4 cells/ml were inoculated into 1 L bottles and grown photoautotrophically in 0.3 HSM in a light-proof, temperature-controlled incubator for at least four 12 h light/12 h dark cycles with the dark phase occurring during the workday. Once cultures reached a concentration of $1 – 2 \times 10^6$ cells/ml, aliquots of 3 ml were transferred to 35 mm Petri

dishes (Corning) and placed in a dark box at the time of the beginning of the dark phase. Cell concentrations were determined using a hemacytometer [37]. For the western blot analyses, cells were harvested in complete darkness at the time of the light pulse from aliquot cultures. For this purpose, 50 ml of the 1 L incubator culture was transferred to a 125 ml Erlenmeyer flask and wrapped in aluminum foil at the time of the beginning of the dark phase during which the light pulse was given. The flask was placed on a shaker at 150 rpm under temperature conditions that were the same as in the incubator, which was 20°C.

4.2 Application of light pulses

Light pulses were applied from an Oriel 150 W solar simulator (Newport) with a dichroic cold mirror (Newport), which reflected only the visible portion of the light onto the culture samples. Before the collimated light beam reached the cultures, it passed through a series of seven beamsplitters (Melles Griot Optics Group), each reflecting approximately 70% of the light beam onto a culture through a 30° diffuser (Edmund Optics) while the remaining 30% was transmitted to the next beam splitter. Thus, the same light beam was able to simultaneously illuminate 7 cultures with graded intensity. Evenness of the light field was confirmed using photographic paper. Between the cold mirror and the first beam splitter, narrow-band interference filters were inserted with center wavelengths from 400 to 700 nm in 20 nm increments (Newport). These filters showed full bandpass width of 10 nm at half-maximum transmittance. A 2.0 or 1.0 absorbance neutral density filter was also inserted to reduce the overall light intensity.

Experimental cultures in the Petri dishes were removed from the dark box and placed into the slots of the light pulse device in complete darkness. Slots were closed with a lid and the light pulse device turned on in order to warm up. The shutter was then opened to expose the cultures for exactly 15 min starting at the time of the designated pulse. For strain CC-124, light pulses were given at 7 h into the dark phase and for strain CC-48 and its derivative RNAi strains at 5 h into the dark phase. At the end of the light pulse, the device was turned off and the cultures returned to the dark box. Finally, all cultures including the controls that did not receive a light pulse were placed into the phototaxis machine in complete darkness 23 h after they were first put into the dark box.

4.3 Monitoring and analysis of circadian phototaxis rhythms

Phototaxis was monitored in an automated fashion for at least five days as previously described [11]. The test light was set to come on every hour for 15 min. The data for these circadian rhythms of phototaxis were analyzed with respect to period and phase using the algorithm previously described [11]. The algorithm was set to calculate the phase at the time of the light pulse and to express it in CT (circadian time) units rather than in absolute hours. A CT unit is equivalent to 1/24th of the free-running period. CT is a generally used unit, because it allows for a better comparison of phases between rhythms that differ in their period. The phase shift of cultures that received a light pulse is expressed with respect to the phase of control cultures that did not receive a light pulse.

4.4 Determining light intensities

A LI-190SA quantum sensor connected to a LI- 250 light meter (LI-COR, Lincoln) was used to measure the light intensities that reached the cultures from the light pulse apparatus. Because the sensitivity of the quantum sensor was limited, it was necessary to extrapolate on some of the lower light intensities when using the narrow-band interference filters. Light intensities either without a neutral density filter or with the 1.0 or 2.0 neutral density filter could be directly measured with the light meter when a narrow-band interference filter was omitted. Light intensities for this white light gave a straight-line response when the logarithm of the intensity was plotted against either the slot number or the strength of the

neutral density filter. When using the narrow-band interference filters, intensities could be directly measured in the first five slots for light that had passed no neutral density filter and for some of the higher light intensity slots with 1.0 and 2.0 neutral density filters in place. These intensities were determined to give the same kind of linear response as white light for measurements above a threshold of 0.02 μmol photon m^{-2} s^{-1}.

Based on these findings of linearity, a model was developed that accounted for the decrease due to neutral density filter and due to slot position: *Log [Intensity] = a * slot + b * ndf + c*, where *slot* is the position of decreasing light intensity in the light pulse device (1 through 7), *ndf* is the decrease in light intensity through the neutral density filter (0, 1 or 2), and *a*, *b and c* are constants. Based on the available light intensity data, a best-fit solution of this model was calculated in Mathematica® (Wolfram) for each narrow-band interference filter and used to extrapolate on the light intensities that were too low for direct measurement. The measured and modeled values were then scaled to correct for measurement characteristics of the light meter at each wavelength, which had been determined by the manufacturer during calibration. Extrapolated values were finally scaled to the light response at 650 nm, because at this wavelength the light meter measurements reflect the ideal quantum response.

4.5 Analysis for action spectrum

Since physiological light responses do not follow a linear relationship with light intensities due to saturation at high light intensities, sigmoidal functions are usually used to model response curves to variations in light intensity. Phase shifts were plotted against the negative logarithm of light intensities and the best-fit equation $y=c/(1+b*e^{-kx})$ was used to model the curve, where *y* is the extent of phase shift, *c* is the maximal response at which light intensity is at saturation, *b* is the horizontal translation factor of the curve, *k* is the rate of response to light intensity, also called the “steepness of the curve”, and *x* is the negative logarithm of the light intensity. In the analysis, *c*, the maximal response under light saturation, was set to −4 CT units. Light intensities required to elicit a −2 CT unit phase shift were then calculated from the best-fit equations by taking the negative antilog of the x-value obtained for a y-value of −2. Statistical analysis was performed using the ANOVA and Chi squared functions in Mathematica®.

4.6 Cloning of RNAi construct and *C. reinhardtii* transformation

Plasmid pCB740 [38] contains the HSP70B gene downstream of the HSP70A/RBCS2 fusion promoter and the ARG7 gene as a marker for *C. reinhardtii*. pCB740 was digested with NheI and EcoRV to remove the HSP70B gene, since the unique NheI site lies directly at the transcription start site and the unique EcoRV site past the 3′ end of the gene, just a few bases ahead of the unique EcoRI site. A ~2.0 kb fragment of plant cryptochrome genomic DNA was removed from pGDS200 [39] by digestion with ScaI and SpeI. Plant cryptochrome was originally named CPH1 (*Chlamydomonas* photolyase homologue 1, AAC37438). It is identical to the annotated protein XP_001701553 in the *C. reinhardtii* genome and most closely related to the cryptochromes of higher plants [16]. The isolated plant cryptochrome fragment starts in exon 1 and ends in exon 4. The fragment was directly inserted in forward orientation into pCB740 in place of the excised HSP70B gene, giving rise to pMY1. A 790 bp fragment of plant cryptochrome cDNA corresponding to the ~2.0 kb fragment of genomic DNA was amplified by PCR from pRCPH101 [13]. An EcoRI site was incorporated into the forward primer and an EcoRV site into the reverse primer (forward: TCGACGAATTCATGCCCCACGAGTTTAAGAC, reverse: CATGCGATATCTGATGGAGCCGATGTGGATC). The amplicon was digested with these two restriction enzymes whereas pMY1 was digested with EcoRI and BsaBI. BsaBI cleaves within the ~2.0 kb plant cryptochrome genomic fragment in pMY1 about 300 bp upstream of its 3′ end. The plant cryptochrome cDNA fragment was ligated into digested

pMY1 just downstream of, and in reverse orientation to, the genomic plant cryptochrome fragment, giving rise to pMY2. The correct junctions in pMY1 and pMY2 were confirmed by sequencing. Thus, the final cryptochrome RNAi gene contains the first three introns of the endogenous gene but, when transcribed and spliced in *C. reinhardtii*, the mRNA will form a 758 bp double-stranded stem with a 15 nt loop.

Transformation of *C. reinhardtii* strain CC-48 was performed using the glass bead method [40]. Cells were grown in TAP medium with arginine to a concentration of 1 to 2×10^6 cells/ml. Cells from a culture volume of 40 ml were resuspended in 1.6 ml autolysin solution prepared according to Harris [37] and incubated 45 min at room temperature with slight shaking. Cells were washed once with 1 ml TAP and resuspended in 0.4 ml TAP. To 1.6 μg of the plasmid pMY2, which had been linearized with XmnI prior to transformation, 300 mg glass beads, 0.3 ml cells, and 0.1 ml 20% PEG 8000 were added and mixed on a vortex for 13 sec at highest speed. TAP medium at a volume of 0.3 ml was immediately added and the solution spread on two TAP plates. Transformants were identified due to their ability to form colonies on these plates without arginine.

4.7 Western blot analysis

The culture samples for all western blot analyses were collected in complete darkness at the time when also a light pulse was given. For each collection, 13.5 ml of culture was transferred to a 15 ml Corning tube, spun in a clinical centrifuge for 90 seconds, and the supernatant poured off. 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, briefly mixed on a vortex and flash frozen in liquid nitrogen before the room lights were turned on and the samples transferred to a −80°C freezer. Cells were thawed and immediately lysed with a sonifier (Vibra-Cell VCX130, Sonics and Materials, Inc.) at 40% amplitude for five 10-sec pulses. Sonicated samples were boiled for 1 min, spun in a microcentrifuge for 2 min, and the supernatant used for western blotting. Protein concentrations were determined spectrophotometrically using the formula:

$$\text{Total protein concentration (mg/ml)} = (1.55 * \text{A}_{280}) - (0.76 * \text{A}_{260}).$$

Equal amounts of total soluble protein were separated on SDS polyacrylamide gels (Mini-PROTEAN 3, Bio-Rad) and transferred to Hybond-C membrane (Amersham Biosciences). Membranes were stained for total protein with Ponceau S (0.2% Ponceau S, 1% acetic acid). After blocking with 3% dried nonfat milk, membranes were incubated with anti-plant cryptochrome antiserum obtained from Gary Small [13] at a 1:5000 dilution. Peroxidase-conjugated anti-rabbit serum (Sigma) was used to detect the primary antibody through chemiluminescence (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, 0.2 mM p-coumaric acid, 0.0000915% hydrogen peroxide) using a digital imaging system (FluorChem HD2, Alpha Innotech). Quantitative analysis of chemiluminescence signals as well as Ponceau S signals was performed using the “band analysis” tool within the FluorChem HD2 software.

Acknowledgments

We particularly thank Gary Small (University of South Dakota, Vermillion) for his anti-plant cryptochrome antiserum and the two plasmids GS200 and pRCPH101 and Michael Schroda for his pCB740 plasmid. This research was supported by the National Institutes of Health, specifically the National Center for Research Resources Grant P20 RR16481 as well as the National Institute of General Medical Sciences Grant 1R15GM076079-01A1, which we gratefully acknowledge.

Abbreviations

CT	circadian time or time under free-running conditions with CT 0 denoting the start of subjective day and CT 12 the start of subjective night
CT unit	$1/24^{th}$ of a circadian rhythm's free-running period
RNAi	RNA interference

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HIGHLIGHTS

- *Chlamydomonas reinhardtii* circadian clock resetting by light was investigated.
- Strain CC-124 is highly sensitive to blue light of 440 nm in this response.
- High sensitivity was also observed to green, red, and potentially UV-A light.
- Only the blue light response deviates from what had been reported for strain CW15.
- Photoreceptor plant cryptochrome likely modulates blue light response negatively.

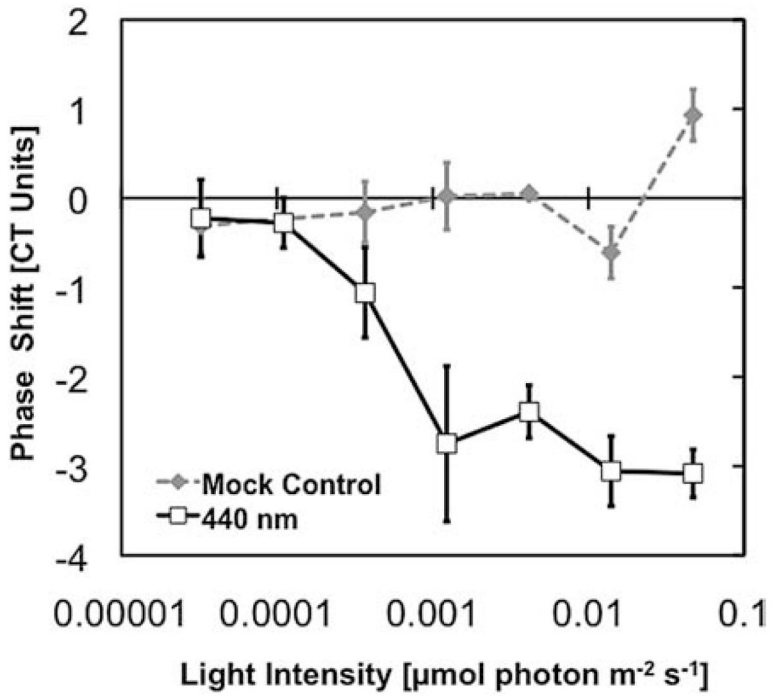

Figure 1. Blue light specifically resets the circadian clock in *C. reinhardtii* strain CC-124

At 7 h into the dark phase of a 12 h light/12 h dark cycle, culture aliquots of strain CC-124 were given a 15 min blue light pulse of 440 nm with various intensities (black solid line) and their circadian rhythms of phototaxis were subsequently determined. Mock control cultures (gray dotted line) were placed into the respective slots of the light pulse apparatus under identical conditions except that the shutter was not opened. Phase shifts are expressed with respect to cultures that were kept in the dark box. A CT unit represents 1/24th of the period under free-running conditions. Each data point represents the average of between two and five independent experiments. Bars indicate the standard error of the mean. Analysis of variance revealed that there is a significant difference in phase shift between cultures exposed to different intensities of 440 nm light (α=0.05), but there is no significant difference in phase shift between mock cultures in different slots of the light pulse device.

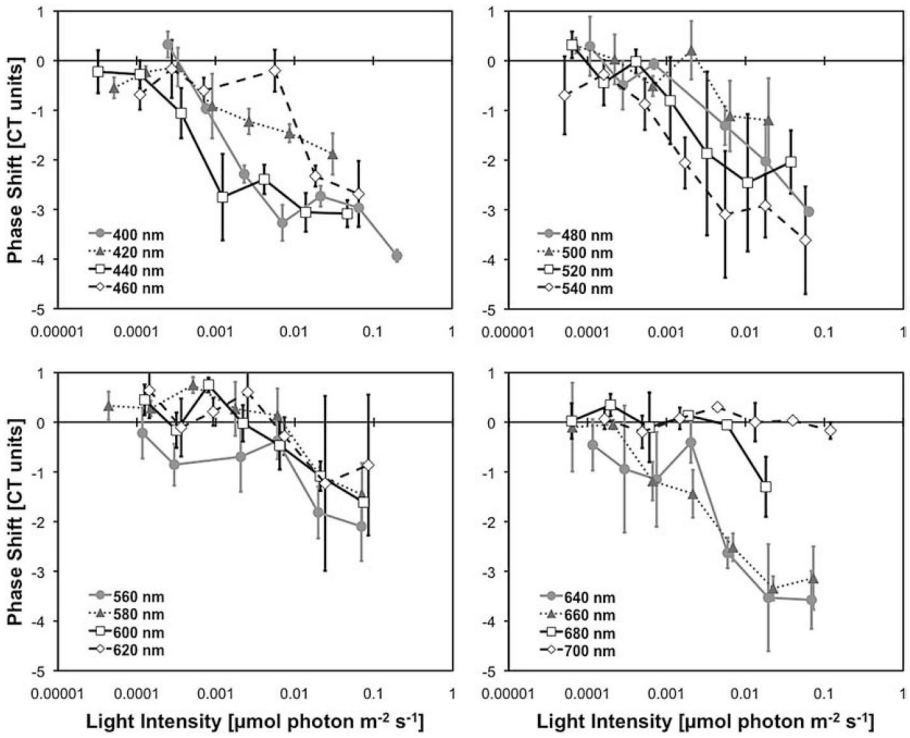

Figure 2. Light intensity response curves for circadian clock resetting at various wavelengths

Cultures were irradiated with 15 min light pulses as described for Figure 1 except that wavelengths every 20 nm between 400 and 700 nm were tested. Phase shifts of the phototaxis rhythm are expressed with respect to cultures kept in the dark. Data points represent the mean of between 2 and 5 independent experiments. Bars indicate the standard error of the mean.

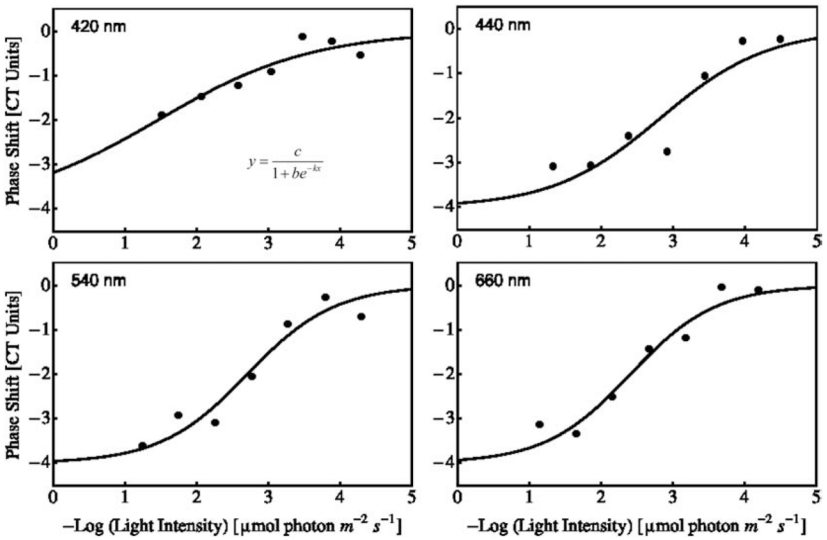

Figure 3. Modeling of light intensity response curves for sensitivity analysis

Data points in the graphs are the same data points for these wavelengths as in Figure 2 and represent the actual phase shift data that were experimentally determined for the circadian rhythm of phototaxis. These data were used to develop a best-fitting equation of the form indicated in the 420 nm graph, where *c* is the phase shift at saturating light intensity, which was set at −4 CT units. The model can be used to calculate the light intensity necessary to cause a phase shift of −2 CT units. R^2 of 420 nm model: 0.961, R^2 of 440 nm model: 0.963, R^2 of 540 nm model: 0.982, R^2 of 660 nm model: 0.983.

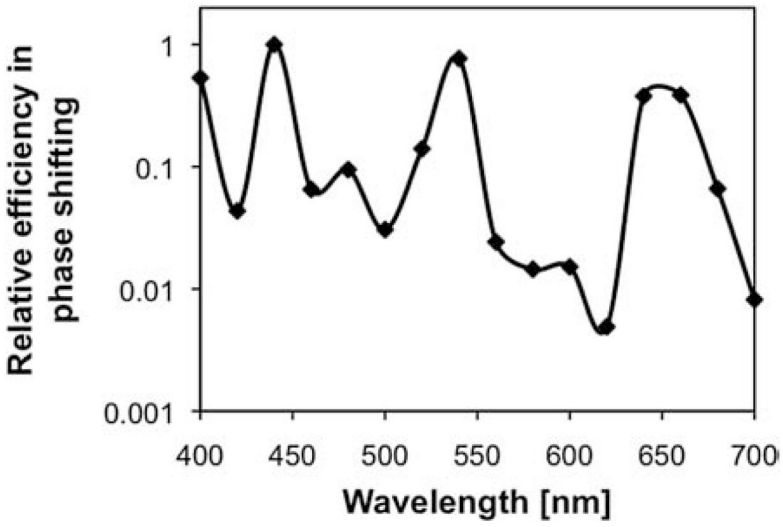

Figure 4. Action spectrum for circadian clock resetting of *C. reinhardtii* strain CC-124

Circadian clock resetting was determined by measuring the degree of phase shift of the circadian phototaxis rhythm exhibited by *C. reinhardtii* CC-124. The light intensity required to cause a phase delay of 2 CT units was calculated from response curve models as exemplified in Figure 3. The reciprocal of this light intensity was normalized against the maximal response at 440 nm, which was set to 1, and plotted on a logarithmic scale against the wavelength.

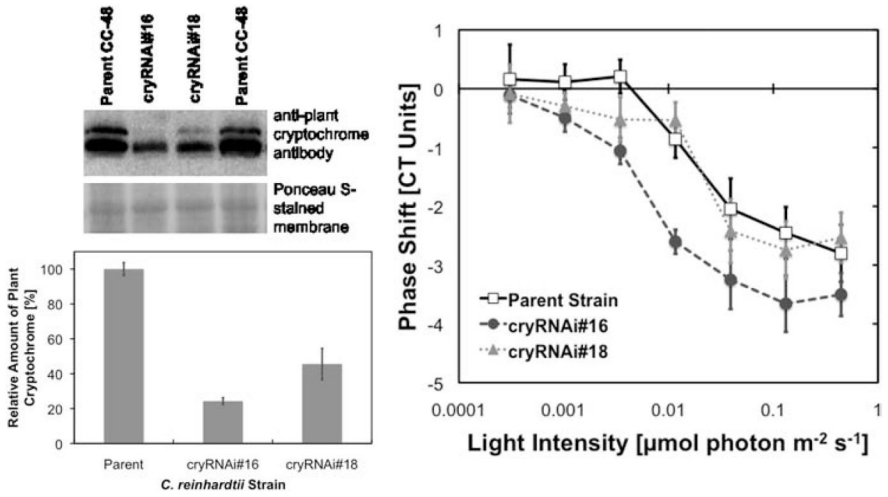

Figure 5. Blue light-induced phase shifts of RNAi strains with knocked-down photoreceptor plant cryptochrome compared to the parent strain CC-48

Upper left panel: Example western blot using an anti-plant cryptochrome antibody and a ~55 kDa uncharacterized protein band of the Ponceau S-stained membrane that served as loading control for normalization. Separation of proteins was achieved on a 7.5% polyacrylamide gel. Plant cryptochrome shows the two previously reported bands with apparent molecular mass of 126 and 143 kDa [13]. Lower left panel: Quantification of western blots relative to the Ponceau S-stained membranes, with the parent strain set at 100%. Each column represents the mean of 3 or 4 independent experiments with 2 technical replicates for each independent experiment. Mean for cryRNAi#16 is 24.3% and for cryRNAi#18 45.6%. Bars indicate the standard error of the mean. Right panel: Phase shifts of the circadian phototaxis rhythm after cultures were exposed to 440 nm light pulses for 15 minutes at 5 h into the dark phase of their 12 h light/12 h dark cycle. Each data point represents the average of at least 3 independent experiments. Bars indicate the standard error of the mean. Analysis of variance revealed that the curve for the cryRNAi#16 strain is significantly different to that of the parent strain CC-48 (α=0.05) but the cryRNA#18 curve is not.

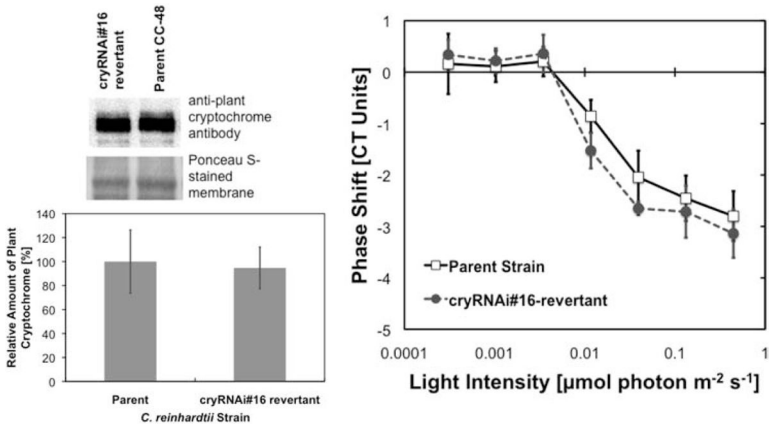

Figure 6. Blue light-induced phase shifts of the revertant cryRNAi#16 strain with about wild-type plant cryptochrome levels

Upper left panel: Example western blot using an anti-plant cryptochrome antibody and a ~55 kDa uncharacterized protein band of the Ponceau S-stained membrane that served as loading control for normalization. Separation of proteins was achieved on a 5–15% acrylamide gradient gel. Lower left panel: Quantification of western blots relative to the Ponceau S-stained membranes, with the parent strain set at 100%. Each column represents the mean of 3 independent experiments. Mean for cryRNAi#16-revertant is 94.7%. Bars indicate the standard error of the mean. Right panel: Phase shifts of the circadian phototaxis rhythm after cultures were exposed to 440 nm light pulses for 15 minutes at 5 h into the dark phase of their 12 h light/12 h dark cycle. Each data point represents the average of at least 3 independent experiments. Bars indicate the standard error of the mean. Analysis of variance revealed that the curve for the cryRNAi#16-revertant strain does not significantly differ from that of the parent strain CC-48.

Table 1

Parameters for the circadian rhythm of phototaxis under free-running conditions exhibited by the various *Chlamydomonas reinhardtii* strains used in this study

Parameters are given for culture samples that did not receive a light pulse but served as dark controls. The acrophase (phase with reference to the peak of phototaxis) is expressed in CT (circadian time), with CT 0 denoting the beginning of subjective day and CT 12 the beginning of subjective night. Each period and phase value represents the average of at least three independent experiments with at least three technical replicates per experiment. The ± standard deviation for each period and phase value is also given.

Strain	Period [h]	Acrophase [CT]
CC-48	26.4 ± 0.9	7.3 ± 0.9
CryRNAi#16	26.2 ± 0.6	6.9 ± 0.8
CryRNAi#16-revertant	26.4 ± 0.4	6.6 ± 0.6
CryRNAi#18	25.5 ± 0.6	7.1 ± 0.8
CC-124	25.6 ± 0.6	6.3 ± 0.7