

# Cryptochromes Are Required for Phytochrome Signaling to the Circadian Clock but Not for Rhythmicity

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**The circadian clock is entrained to the daily cycle of day and night by light signals at dawn and dusk. Plants make use of both the phytochrome (phy) and cryptochrome (cry) families of photoreceptors in gathering information about the light environment for setting the clock. We demonstrate that the phytochromes phyA, phyB, phyD, and phyE act as photoreceptors in red light input to the clock and that phyA and the cryptochromes cry1 and cry2 act as photoreceptors in blue light input. phyA and phyB act additively in red light input to the clock, whereas cry1 and cry2 act redundantly in blue light input. In addition to the action of cry1 as a photoreceptor that mediates blue light input into the clock, we demonstrate a requirement of cry1 for phyA signaling to the clock in both red and blue light. Importantly, *Arabidopsis cry1 cry2* double mutants still show robust rhythmicity, indicating that cryptochromes do not form a part of the central circadian oscillator in plants as they do in mammals.**

## INTRODUCTION

The circadian clock controls physiological and biochemical processes essential to the lives of all organisms. Possession of an endogenous oscillator allows an organism to anticipate dawn and dusk and to prepare for the marked environmental changes associated with these transitions (Dunlap, 1999). In mammals, the sleep/wake cycle and fluctuation in body temperature are both under the control of the circadian clock; in insects, processes such as larval eclosion can be timed to occur at the optimum time of day, giving an adaptive advantage important for survival; and in plants, the processing of the photosynthetic machinery begins just before dawn in preparation for the light-harvesting reactions.

Processes controlled by the circadian clock continue to oscillate with a period of ~24 hr even in constant environmental conditions, thereby implicating the involvement of an endogenous oscillator. To be of use to the organism, however, this oscillator must first be synchronized to the environmental day/night cycle. The two most striking changes at dawn and dusk are in light and temperature, and both of these environmental factors are capable of entraining the circadian clock (Devlin and Kay, 2000a).

Recent studies have revealed much about the nature of the photoreceptors responsible for light input to the clock. In plants, light plays a crucial role in the regulation of development at every stage of the life history, and several plant

photoreceptors have been well characterized (Johnson et al., 1994; Whitelam and Devlin, 1998). Of these, the phytochrome (phy) family, absorbing in the red region of the spectrum, and the cryptochrome (cry) family, absorbing in the blue, both mediate light input to the clock (Somers et al., 1998).

Phytochrome regulates a range of developmental processes in response to red and far-red light, including seedling establishment, shade avoidance, and transition to flowering. The phytochromes consist of a protein moiety of ~124 kD with a covalently attached linear tetrapyrrole chromophore. The phytochrome molecule exists in two photo-interconvertible forms—an inactive, red-absorbing form (Pr) and an active, far-red-absorbing form (Pfr) (Quail et al., 1995). Although the strongest absorption peak of phytochromes is in the red or far-red region of the spectrum, another important absorption peak occurs in the blue region; consequently, phytochromes have been implicated in mediating several responses to blue light as well (Whitelam et al., 1993; Casal and Mazzella, 1998; Neff and Chory, 1998).

Higher plants contain multiple phytochromes, the product of a multigene family. *Arabidopsis* has five phytochromes, phyA to phyE, and the function and modes of action of these distinct phytochrome species have been the subject of much recent investigation. Physiological studies of mutants deficient in one or more phytochromes have indicated both unique and overlapping roles for the various phytochrome family members (Whitelam et al., 1998).

Plant cryptochromes, which were discovered more recently (Ahmad and Cashmore, 1993; Hoffman et al., 1996), specifically mediate responses to blue light, showing a strong absorption peak in the blue region of the spectrum.

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However, because they also show a slight peak of absorption in the green region, cryptochromes have also been implicated in responses to green light (Lin et al., 1995; Whitelam, 1995).

The N termini of cryptochrome molecules show strong homology with the type II photolyase DNA repair enzymes. They possess two noncovalently linked chromophores, a light-harvesting pterin and a catalytic flavin (Cashmore et al., 1999). Arabidopsis contains two cryptochromes, cry1 and cry2, each with unique C-terminal extensions. Analysis of mutants deficient in one or both cryptochromes has revealed distinct roles for each (Cashmore et al., 1999).

The mode of action of the photoreceptors remains unclear, but several recent pieces of evidence have begun to shed light on it. Phytochrome and cryptochrome molecules linked to fluorescent protein tags have been used to study the subcellular localization of these molecules in living cells (Cashmore et al., 1999; Guo et al., 1999; Kircher et al., 1999; Yamaguchi et al., 1999). Both the phytochromes and the cryptochromes are localized in the cell nucleus; the phytochromes display a light-dependent nuclear localization (Kircher et al., 1999), whereas cryptochromes are constitutively nuclear (Cashmore et al., 1999; Guo et al., 1999; Mas et al., 2000). In addition, several molecules interacting with phyA and phyB have recently been identified by yeast two-hybrid screening. Phytochrome-interacting factor (PIF3; a basic helix-loop-helix transcription factor) (Ni et al., 1998), phytochrome kinase substrate (Fankhauser et al., 1999), and nucleoside diphosphate kinase (Choi et al., 1999) have all been identified as binding to phytochrome.

Interestingly, both cry1 and cry2 interact with phyA in vitro. Furthermore, in vivo evidence suggests that cry1 is phosphorylated in response to red light (Ahmad et al., 1998). The significance of this interaction has proved elusive, although missense mutations in cry1 have a slight, dominant negative effect on phyA signaling. Nonetheless, no decrease in phytochrome signaling has been demonstrated in a cry1 null mutant.

After their discovery in plants, cryptochromes were also discovered in insects and mammals (Cashmore et al., 1999). Cryptochrome mediates light input to the clock in *Drosophila*, exhibiting a light-dependent interaction with one of the molecules making up the clock mechanism (Ceriani et al., 1999). In contrast, the mammalian cryptochromes form part of a negative feedback loop that is itself the central oscillator (Kume et al., 1999). For example, mice deficient in both mCRY1 and mCRY2 are completely arrhythmic (van der Horst et al., 1999). Whether the mammalian cryptochromes also play any role in light-based resetting of the clock in mammals remains uncertain (Devlin and Kay, 1999, 2000a).

In Arabidopsis, as in other diurnal organisms, the length of the clock period decreases with increasing light intensity, a phenomenon known as Aschoff's rule (Aschoff, 1979). Using the *CAB2::LUC* construct to follow the circadian oscillation in *CAB2* transcription, Millar et al. (1992, 1995) demonstrated that the period of oscillation decreases from a range of 30 to 36 hr in darkness to ~24 hr in light. Furthermore, by

generating fluence rate response curves to examine the effect of increasing intensities of red or blue light on period length in photoreceptor mutants, we demonstrated previously that phyA and phyB are involved in red light input to the clock and that phyA and cry1 are involved in blue light input to the clock (Somers et al., 1998).

Here, we have analyzed circadian photoperception in double and triple phytochrome and cryptochrome mutant combinations. We demonstrate that phyD and phyE also mediate red light input to the clock. We show that phyA and phyB act additively to regulate red light input to the clock, whereas cry1 and cry2 act redundantly in blue light input to the clock. Importantly, we demonstrate that cry1 and cry2 do not form part of the clock machinery itself in Arabidopsis, as they do in mammals. However, we demonstrate a novel role for cryptochrome in red light signaling downstream of phyA, specifically in light input to the clock, revealing a possible significance to the previously observed interaction between phyA and cryptochrome in vitro.

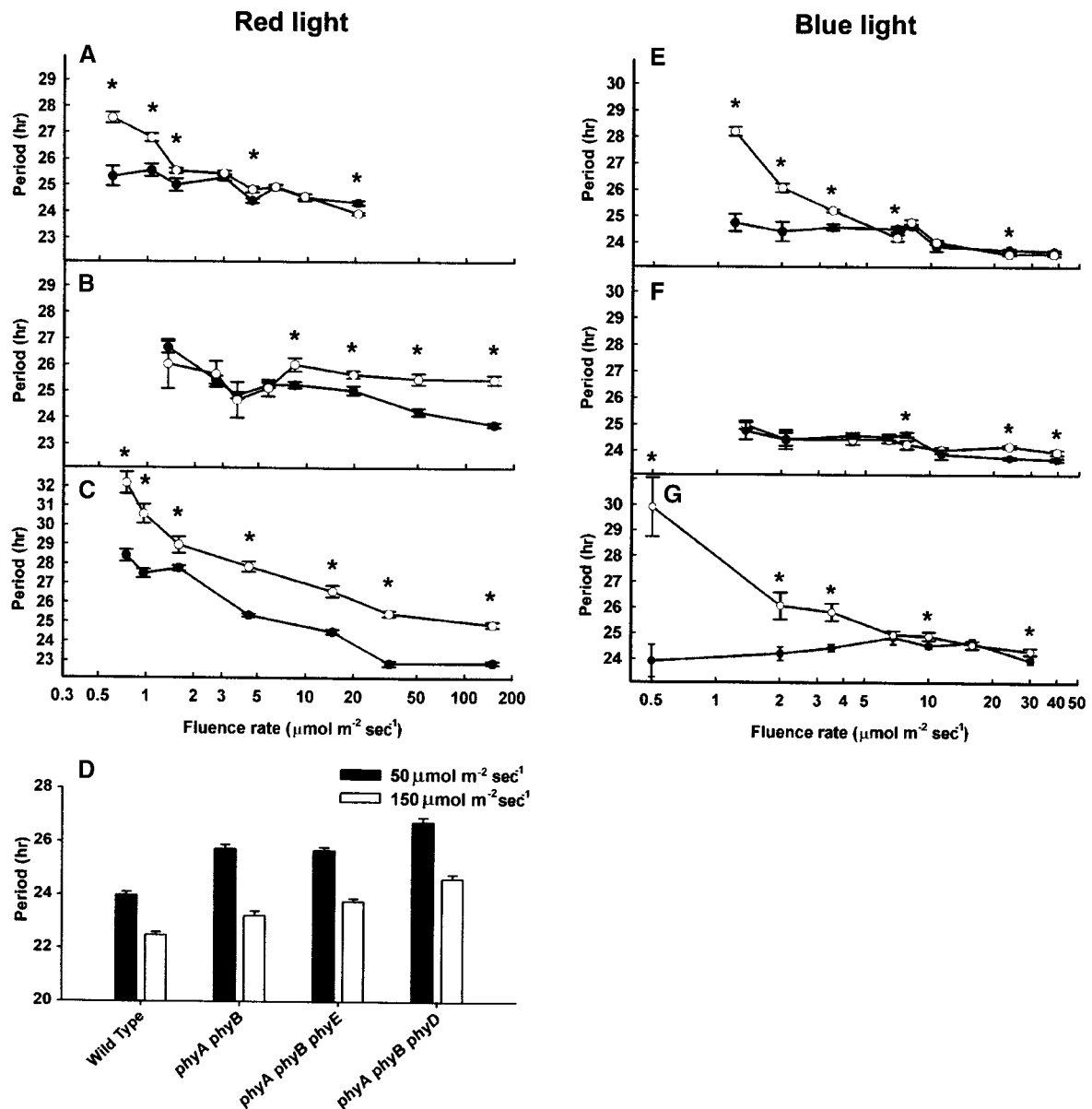
## RESULTS

### phyA, phyB, phyD, and phyE Act Additively in Red Light Input to the Clock

Wild-type and phytochrome mutant seedlings were entrained from germination for 6 days in 12-hr-white-light/12-hr-dark cycles, after which they were transferred to continuous red light at various different intensities. Circadian oscillation in the expression of *CAB2::LUC* was then monitored for 5 days more. As reported previously (Somers et al., 1998), in wild-type seedlings of Arabidopsis, the free running period of *CAB2::LUC* expression shortens with increasing intensity of red light. The *phyA* mutant, which is deficient in perception of low-fluence-rate red light, displays a longer than wild-type period under these conditions (Figure 1A). In contrast, the *phyB* mutant is deficient in perception of high fluence rates of red light (Figure 1B).

We examined the effect of fluence rate on length of period in *phyA phyB* double mutant seedlings. The *phyA phyB* double mutant displayed a long period across the whole range of red light fluence rates, indicating an additivity between phyA and phyB in red light control of period length (Figure 1C); that is, the effects attributable to the *phyA* and *phyB* mutations are combined in the double mutant.

The less abundant phytochromes phyD and phyE display a conditional redundancy with phyB (Devlin et al., 1998, 1999). Consistent with this, we observed a wild-type response to red light in the *phyD* and *phyE* monogenic mutants (data not shown). We then compared the roles of phyD and phyE in light input to the clock in the background of the *phyA phyB* double mutant. The highest two fluence rates of light were used for this comparison because too little *CAB2* was expressed for detection in the triple mutants at low flu-



**Figure 1.** Effect of Light Intensity on Period Length of the Circadian Rhythm of *CAB2::LUC* Bioluminescence in Wild-Type and Phytochrome-Deficient Seedlings.

Seedlings were germinated and grown in 12-hr-white-light/12-hr-dark cycles for 6 days and then transferred to constant red or blue light at the fluence rates indicated for 5 days. Values shown are means ( $\pm$ SE) for wild type (closed circles) and mutants (open circles).

(A) Wild type and *phyA* mutant in red light.

(B) Wild type and *phyB* mutant in red light.

(C) Wild type and *phyA phyB* double mutant in red light.

(D) Mean period length for wild-type, *phyA phyB*, *phyA phyB phyE*, and *phyA phyB phyD* mutant seedlings in red light of 50  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  (solid bars) and 150  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  (open bars).

(E) Wild type and *phyA* mutant in blue light.

(F) Wild type and *phyB* mutant in blue light.

(G) Wild type and *phyA phyB* double mutant in blue light.

Asterisk,  $P < 0.01$  (Student's two-tail heteroscedastic *t* test).

ence rates. The *phyA phyB phyD* triple mutant displayed a slightly longer *CAB2::LUC* expression period than did the *phyA phyB* double mutant at high fluence rates of red light, indicating that *phyD* plays a role in the red light control of period length (Figure 1D). The *phyA phyB phyE* triple mutant was less responsive than the *phyA phyB* double mutant to red light at the highest light intensity used, which suggests a small but important role for *phyE* in control of period length by input of red light (Figure 1D). Both the *phyA phyB phyD* and *phyA phyB phyE* triple mutants retained a response to an increased fluence rate of red light, an indication that other phytochromes may also be active.

### **phyB Plays No Role in Blue Light Input to the Clock**

The effect of fluence rate of blue light on the length of the free running period of the *CAB2::LUC* rhythm was examined in wild-type and phytochrome mutant seedlings. Seedlings were entrained in white light/dark cycles before transfer to blue light of different intensities for monitoring the circadian oscillation in *CAB2::LUC* expression. As reported previously (Somers et al., 1998), blue light shortens the free running period in wild-type seedlings from the dark period range of 30 to 36 hr (Millar et al., 1995). The *phyA* mutant was deficient in perception of low-fluence-rate blue light, displaying a longer than wild-type period length in those conditions. The *phyB* mutant, however, displayed a wild-type response to blue light for shortening the expression period (Figures 1E and 1F). The *phyA phyB* double mutant showed a lengthening of period at low fluence rates that was consistent with the loss of *phyA* but indicating no *phyB* function in blue light (Figure 1G).

### **cry1 and cry2 Act Redundantly in Blue Light Input to the Clock and Are Not Essential for Circadian Rhythmicity**

The length of the free running period of the *CAB2* transcription rhythm was examined in *cry1*, *cry2*, and *cry1 cry2* mutant seedlings in blue light. As previously observed (Somers et al., 1998), the *cry1* mutant displayed a longer period than wild type at both low and high fluence rates of blue light but showed a wild-type period length at intermediate fluence rates of blue light. The *cry2* mutant displayed a wild-type response across the whole range of blue light fluence rates, except for a slight decrease relative to wild type at the fluence rate of  $2 \mu\text{mol m}^{-2} \text{sec}^{-1}$  (Figures 2A and 2B). The *cry1 cry2* double mutant exhibited a long period of *CAB2::LUC* oscillation in all fluence rates of blue light, indicating a role for both *cry1* and *cry2* in perception of blue light in the control of the period length of the endogenous clock. *cry1* and *cry2* act with complete redundancy at intermediate fluence rates of blue light in the control of circadian period; that is, loss of both photoreceptors is required to see a change in phenotype over this range (Figure 2C). A role for *cry2* at low and high fluence rates is also indicated because the *cry1*

*cry2* double mutant shows a longer period than does the *cry1* mutant at these fluence rates.

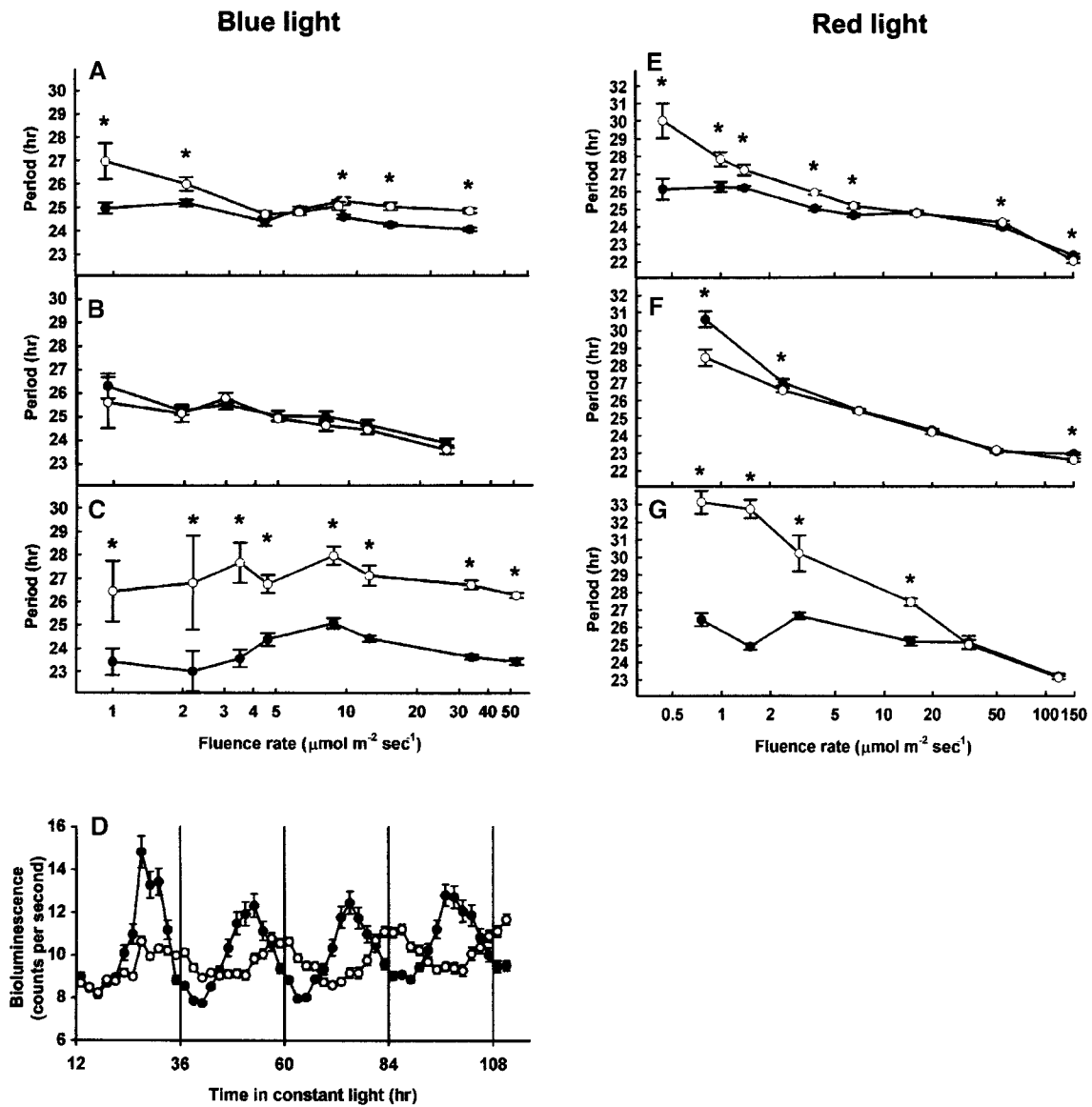
The oscillation of *CAB2::LUC* in the *cry1 cry2* double mutant also has a slightly smaller amplitude at all fluence rates, consistent with a role for cryptochrome in the maintenance of a strong expression of *CAB2* as well as in light input to the clock (Figure 2D).

Importantly, a robust rhythm of *CAB2::LUC* expression is still observed in the absence of both *cry1* and *cry2* (Figure 2D)—in sharp contrast to the situation in mammals, where loss of both cryptochromes results in arrhythmicity (van der Horst et al., 1999). This result, therefore, indicates a clear divergence in the organization of the mammalian and plant circadian machinery.

### **cry1 and cry2 Act in Red Light Input to the Clock**

The period length of the rhythm of *CAB2* transcription was examined in the *cry1*, *cry2*, and *cry1 cry2* mutants in red light. The *cry1* mutant displayed a deficiency in response to low-fluence-rate red light, showing a longer than wild-type period under these conditions, but demonstrated a wild-type period length in intermediate- and high-fluence-rate red light (Figure 2E). The *cry2* mutant displayed a wild-type period length at all fluence rates of red light, except for a slightly shorter period at the lowest fluence rate examined (Figure 2F). The *cry1 cry2* double mutant showed a long period of *CAB2::LUC* expression in both low and intermediate fluence rates of red light, and this effect extended to higher fluence rates than those at which *cry1* deficiency alone is effective (Figure 2G). Furthermore, the effect of losing both *cry1* and *cry2* was a greater lengthening of the circadian period at low fluence rates than that seen with loss of *cry1* alone (Figure 2G). This is consistent with a conditional redundancy in the action of *cry1* and *cry2* in red light signaling to the clock, the effect of *cry2* deficiency being apparent only in a *cry1* mutant background.

Because cryptochromes show no absorption peak in the red region of the spectrum (Lin et al., 1995), *cry1* and *cry2* may be acting as signal transduction components downstream of phytochrome, most notably in the range of fluence rates perceived by *phyA*. In fact, cryptochrome appears to be essential for *phyA* signaling to the clock. To test whether this is the case for other phytochrome-mediated responses to red light, we examined in phytochrome and cryptochrome mutants the effect of red light on inhibition of hypocotyl elongation during deetiolation and seedling establishment. One-day-old etiolated seedlings were grown in darkness or at one of several fluence rates of red light for 3 days, after which hypocotyl lengths were determined. Wild-type seedlings of *Arabidopsis* showed increasing inhibition of hypocotyl elongation with increasing fluence rate (Figure 3). Seedlings of the *phyA* mutant showed less inhibition of hypocotyl elongation at low fluence rates of red light, consistent with the range of fluence rates over which *phyA* acts in



**Figure 2.** Effect of Blue Light Intensity on Period Length of the Circadian Rhythm of *CAB2::LUC* Bioluminescence in Wild-Type and Cryptochrome-Deficient Seedlings.

Seedlings were germinated and grown in 12-hr-white-light/12-hr-dark cycles for 6 days and then transferred to constant blue or red light at the fluence rates indicated for 5 days. Values shown are means ( $\pm$ SE) for wild type (closed circles) and mutants (open circles).

(A) Wild type and *cry1* mutant in blue light.

(B) Wild type and *cry2* mutant in blue light.

(C) Wild type and *cry1 cry2* double mutant in blue light.

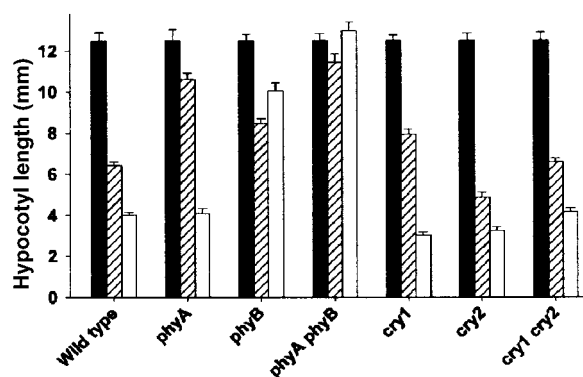
(D) Circadian rhythm of *CAB2::LUC* bioluminescence in wild-type and *cry1 cry2* double mutant seedlings in blue light at 53  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ .

(E) Wild type and *cry1* mutant in red light.

(F) Wild type and *cry2* mutant in red light.

(G) Wild type and *cry1 cry2* double mutant in red light.

Asterisk,  $P < 0.01$  (Student's two-tail heteroscedastic *t* test).



**Figure 3.** Effect of Red Light Intensity in Hypocotyl Length in Wild-Type and Photoreceptor-Deficient Seedlings.

One-day-old dark-grown wild-type and mutant seedlings were either maintained in darkness (solid bars) or transferred to low-fluence-rate red light ( $0.3 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ; striped bars) or high-fluence-rate red light ( $30 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ; open bars) for 3 days, after which hypocotyl lengths were measured. Mean ( $\pm$ SE) hypocotyl lengths are normalized to dark-grown wild-type seedlings.

light input to the circadian clock (Figure 3). Seedlings of the *phyB* mutant, in contrast, showed decreased inhibition of hypocotyl elongation at high fluence rates of red light, consistent with the range of fluence rates over which *phyB* acts in light input to the circadian clock (Figure 3). The *phyA phyB* double mutant showed no noticeable response to red light at either low or high fluence rates, indicating that *phyA* and *phyB* account for the vast majority of the inhibition of hypocotyl elongation in red light (Figure 3). The *cry1* and *cry2* mutants behave very similarly to wild-type seedlings for inhibition of hypocotyl elongation at all fluence rates of red light (Figure 3). The *cry1* mutant shows slightly longer hypocotyls than do wild-type seedlings in low fluence rates of red light, but this effect is much smaller than the effect observed in the absence of *phyA*. This indicates that cryptochrome is not essential for the *phyA* signaling in red light that controls inhibition of hypocotyl elongation (Figure 3).

#### Mutations in *phyA* and *cry1* Are Epistatic in Their Effects on Light Input to the Circadian Clock

To test the hypothesis that *cry1* may act downstream of *phyA* in light input to the clock in all wavelengths of light, as opposed to only in red light, we examined the fluence rate response curve for the length of the free running period of the *CAB2::LUC* rhythm in white light in *phyA*, *cry1*, and *phyA cry1* mutants. Wild-type seedlings of *Arabidopsis* showed a decrease in period length in response to increasing the white light fluence rate (Figure 4A). The *phyA* and *cry1* mutants were both deficient in response to low fluence rates of white light (Figures 4A and 4B), indicating that both *phyA* and *cry1*

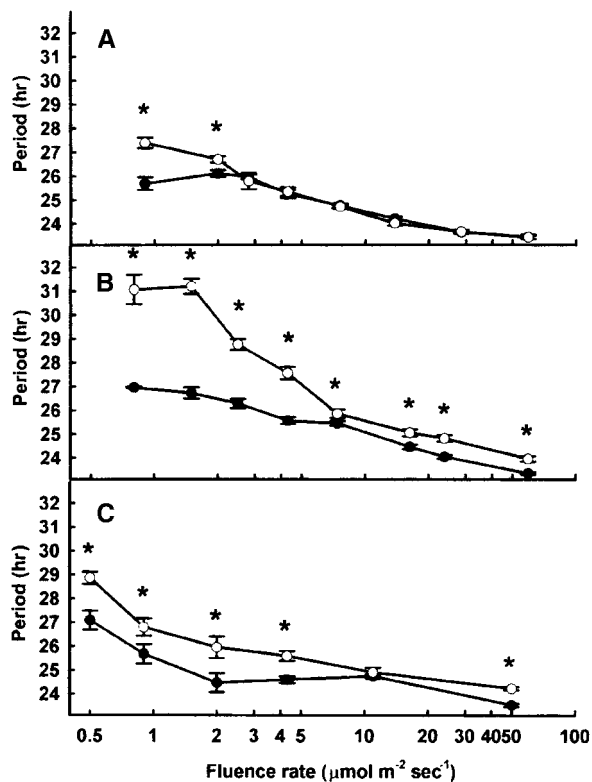
are required for normal shortening of the period in response to low-fluence-rate white light, even though each alone is capable of acting as a photoreceptor under these conditions. At higher fluence rates of white light, the *phyA* mutant showed a wild-type period length consistent with the phenotype of *phyA* in monochromatic red or blue light. The *cry1* mutant showed a deficiency in response to high-fluence-rate white light (Figure 4B), consistent with the decreased sensitivity to high-fluence-rate blue light in the *cry1* mutant.

The *phyA cry1* double mutant showed a deficiency in response to low-fluence-rate white light (Figure 4C). The *phyA* and *cry1* mutations displayed epistasis at low fluence rates of white light; that is, no additivity was observed between the *phyA* and *cry1* mutations. Curiously, the magnitude of the effect of loss of both *phyA* and *cry1* at low fluence rates is less than that seen for the loss of *cry1* alone. However, the lack of additivity between the *phyA* and *cry1* mutations is consistent with the hypothesis that *cry1* acts downstream of *phyA* in light signaling to the clock. The *phyA cry1* double mutant showed a wild-type response to intermediate fluence rates of white light ( $10 \mu\text{mol m}^{-2} \text{sec}^{-1}$ ) but a deficient response to high-fluence-rate white light; this is consistent with a role of *cry1* as a blue light photoreceptor in its own right at high fluence rates (Figure 4C).

Interestingly, the range of fluence rates over which *cry1* acts to mediate both red and white light input to the clock extends farther than the range over which *phyA* acts (Figures 2E and 4B). In both red and white light, *cry1* disrupts light input to the clock over some of the range of fluence rates in which *phyB* acts to mediate light to the clock. Thus, to some extent, *cry1* may act downstream of *phyB* signaling, which may become important at the low end of the range of fluence rates over which *phyB* acts to mediate light input to the clock.

#### Loss of Phytochromes or Cryptochromes Does Not Affect the Circadian Clock in Darkness

The length of the free running period of the circadian clock in wild-type, *phyA phyB* double mutant, and *cry1 cry2* double mutant seedlings was examined in darkness. The circadian rhythm of *CCR2* transcript abundance (Krebs and Simon, 1997) was used as a marker because the expression of *CAB2* decreases rapidly in the absence of light. Seedlings were entrained in 12-hr-white-light/12-hr-dark cycles for 6 days and then transferred to constant darkness. Batches of seedlings were harvested after 72 hr in darkness and then at 3-hr intervals thereafter for another 33 hr. RNA was extracted from these seedlings, and after electrophoresis and transfer to nitrocellulose filters, samples were probed with a radiolabeled *CCR2* probe. If the lengths of the circadian period in darkness were identical for wild-type and photoreceptor-mutant seedlings, the rhythms of *CCR2* expression in both mutant and wild type would still be in phase over the course of the assay, despite the free running for 72 hr before sampling. Over the



**Figure 4.** Effect of White Light Intensity on Period Length of the Circadian Rhythm of *CAB2::LUC* Bioluminescence in Wild-Type and Phytochrome- and Cryptochrome-Deficient Seedlings.

Seedlings were germinated and grown in 12-hr-white-light/12-hr-dark cycles for 6 days and then transferred to constant red light at the fluence rates indicated for 5 days. Values shown are means ( $\pm$ SE) for the wild type (closed circles) and mutants (open circles).

(A) Wild type and *phyA* mutant.

(B) Wild type and *cry1* mutant.

(C) Wild type and *phyA cry1* double mutant.

Asterisk,  $P < 0.01$  (Student's two-tail heteroscedastic  $t$  test).

course of the 33-hr interval of the assay, the rhythm of *CCR2* expression in wild type and in the phytochrome and cryptochrome double mutants coincided almost exactly, suggesting that there was no difference in period length between the different genotypes over the preceding 72 hr in darkness (Figure 5).

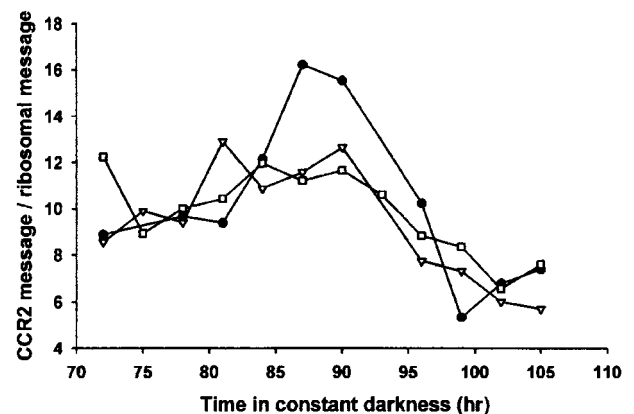
## DISCUSSION

### Distinct and Overlapping Roles for Phytochromes and Cryptochromes in Light Input to the Circadian Clock

Analysis of *Arabidopsis* mutants deficient in multiple photoreceptors has revealed complex interactions between the

various photoreceptors that mediate light input to the circadian clock. The action of *phyA* and *phyB* in red light is additive: *phyA* mediates low-fluence-rate red light input to the clock, whereas *phyB* mediates high-fluence-rate red light input to the clock. The *phyA phyB* double mutant behaves exactly as would be predicted from the combination of the two monogenic mutant phenotypes, pointing to a plasticity in the recruitment of these different photoreceptors for light input to the clock in different conditions. Roles for *phyD* and *phyE* in the perception of high-fluence-rate red light are also indicated. Importantly, the *phyA phyB phyD* and *phyA phyB phyE* triple mutants still show a strong response to an increase in the fluence rate, suggesting action by other phytochromes. Phytochrome-mediated responses have previously been observed in the *phyA phyB phyD* and *phyA phyB phyE* triple mutants in the shade avoidance response (Devlin et al., 1998, 1999). Generation of the *phyA phyB phyD phyE* quadruple mutant will be required to determine whether these four phytochromes alone can account for red light input to the clock or whether the remaining response in the triple mutants represents the action of *phyC*.

As was previously demonstrated (Somers et al., 1998), although the *phyA* mutant shows a deficiency in low-fluence-rate blue light input to the clock, the *phyB* mutant shows no defect in blue light input to the clock. Often a conditional redundancy is observed between *phyA* and *phyB* in which the action of one photoreceptor is able to compensate for the loss of another. In such cases, the effects of a mutation causing loss of one photoreceptor are apparent only in the absence of the other (Devlin et al., 1996). The



**Figure 5.** Circadian Rhythm of *CCR2* Expression in Wild-Type, *phyA phyB*, and *cry1 cry2* Seedlings.

Seedlings were germinated and grown in 12-hr-white-light/12-hr-dark cycles for 6 days and then transferred to constant darkness. Tissue was harvested after 72 hr and then at 3-hr intervals thereafter for an additional 33 hr. Total RNA was extracted, and *CCR2* RNA was quantified as described in Methods. Wild-type, closed circles; *phyA phyB*, open triangles; *cry1 cry2*, open squares.

response of the *phyA phyB* double mutant to blue light reveals no such conditional redundancy between *phyA* and *phyB* action in blue light, thus indicating that *phyB* is unlikely to play any role in blue light input to the clock.

The response of the *cry1 cry2* double mutant reveals a conditional redundancy between *cry1* and *cry2* in blue light input to the clock. The action of *cry2* in perception of intermediate fluence rates of blue light is apparent only in the absence of *cry1*, which otherwise compensates for loss of *cry2* in the *cry2* mutant.

### Cryptochromes Are Not Required for Circadian Rhythmicity in *Arabidopsis*

The *cry1 cry2* double mutant shows a strong circadian rhythm of *CAB2::LUC* expression in blue light. This is distinct from the phenotype of the mouse *mCRY1<sup>-/-</sup> mCRY2<sup>-/-</sup>* double mutant, which is arrhythmic in constant conditions (van der Horst et al., 1999). The mouse cryptochromes *mCRY1* and *mCRY2* form part of a transcriptional feedback loop that makes up the central circadian oscillator in mammals, and loss of both *mCRY1* and *mCRY2* stops the clock (Kume et al., 1999). The plant cryptochromes clearly do not act within the clock mechanism itself, which means that their role is distinct from that of the mammalian cryptochromes. This is consistent with a phylogenetic analysis of the animal and plant cryptochromes that suggests that cryptochromes arose independently in plants and animals (Cashmore et al., 1999). This analysis suggests that the plant cryptochromes diverged from the type II photolyases before the divergence of plants and animals, whereas the animal cryptochromes diverged more recently from the 6-4 photolyases. Animals are therefore presumed subsequently to have lost the cryptochrome sequences related to the type II photolyases (Cashmore et al., 1999). Intriguingly, however, despite their divergent origins and divergent roles, both animal and plant cryptochromes are associated with the circadian clock. Cryptochromes in insects are particularly interesting in that the role of the *Drosophila* cryptochromes is closer to that of the plant cryptochromes. Although *dCRY* interacts directly with the components of the central oscillator, it is not essential for the running of the clock, and its role is purely one of light input to the clock (Ceriani et al., 1999). Apparently, the recruitment of cryptochrome as the photoreceptor that mediates light input to the clock in insects and plants evidences considerable convergent evolution.

### *cry1* Is Required for *phyA* Signaling to the Clock

The response of the *cry1* monogenic mutant and the *cry1 cry2* double mutant to red light suggests roles for *cry1* and *cry2* as signal transduction components downstream of *phyA* and, to some extent, downstream of *phyB* in red light input to the clock.

Despite the fact that cryptochrome shows no peak of absorption in the red region of the spectrum (Lin et al., 1995), the *cry1* mutant fails to perceive low fluence rates of red light that, in wild-type seedlings, mediate a shortening of the period of the clock. Furthermore, although the *cry2* mutant shows a wild-type phenotype in red light, the *cry1 cry2* double mutant displays a greater disruption of light signaling to the clock in low-fluence-rate red light than is seen in the *cry1* monogenic mutant.

The effect of cryptochrome deficiency in red light input to the clock extends to higher fluence rates than the range over which *phyA* acts. Loss of the cryptochromes in red light also affects the lower end of the range of fluence rates over which *phyB* acts in light input to the clock. This suggests that some *phyB* signaling may also occur through cryptochrome. This effect is apparent only at the lower end of the range of fluence rates within which *phyB* acts, indicating that cryptochrome participation is not the major mechanism for *phyB* signaling; this finding may even represent some nonspecific action of *phyB* in the *phyA* signal transduction pathway. *phyB* can act in light input independently of cryptochrome, as evidenced by the normal response of the cryptochrome mutants at the higher end of the range of red light fluence rates over which *phyB* acts.

The fact that *cry1* may be necessary for *phyA* signaling to the clock was further investigated. Both the *phyA* and *cry1* monogenic mutants displayed a deficiency in the perception of low fluence rates of white light. Both phytochromes and cryptochromes act strongly as photoreceptors in white light (Koornneef et al., 1980; Johnson et al., 1994), and each is involved in light input to the clock under these conditions. The failure of each of these two photoreceptors to compensate for loss of the other suggests that they are not acting independently. Consistent with this, the *phyA cry1* double mutant behaves like the *cry1* mutant in that they both show a deficiency in response to white light over an almost identical range of fluence rates. The *phyA cry1* double mutant shows, if anything, less of a deficiency in white light input to the clock than is seen in the *cry1* mutant, although the reason for this remains unclear. Interestingly, the *phyA cry1* double mutant demonstrates no additivity between the effects of the *phyA* and *cry1* mutations, as might be expected for the combination of mutations within two components of a single pathway.

The *cry1* mutant also showed a deficiency in perception of high-fluence-rate white light, consistent with the role of *cry1* in perception of high fluence rates of blue light.

Ahmad et al. (1998) recently demonstrated that *phyA* directly interacts with *cry1* and *cry2* in vitro and that *phyA* mediates a red light-dependent phosphorylation of *cry1*. The action of *cry1* and *cry2* downstream of *phyA* provides the first evidence of a possible relevance for this interaction. *phyA* acts as a photoreceptor in this interaction, whereas *cry1* and *cry2* are acting purely as signal transduction components. That the *cry1* allele used was null eliminates the possibility of any dominant negative effects by mutant cryp-

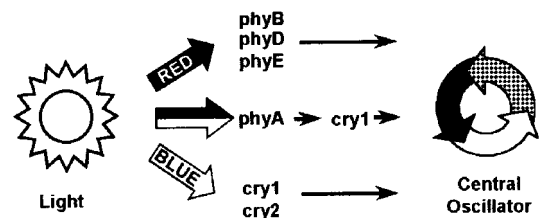


tochrome on phyA signaling. The requirement for cryptochrome in phyA signaling in red light is independent of any light excitation of cry1 or cry2 because cryptochrome shows no substantial absorbance at red wavelengths (Lin et al., 1995). Cryptochromes have two chromophores, a pterin and a flavin (Cashmore et al., 1999), as do the photolyases. In type II photolyases, absorption of a photon of light by the light-harvesting pterin causes transfer of an electron to the catalytic flavin, which in turn donates this electron in a reaction that breaks any pyrimidine dimers that may have formed as a result of DNA damage (Cashmore et al., 1999). Cryptochrome has been proposed to make use of such redox signaling to trigger plant responses to light; perhaps the absorbance of light by phyA is also able to initiate this redox signal within the cryptochrome molecule. In addition, both phytochromes and cryptochromes are nuclear localized in light (Cashmore et al., 1999; Guo et al., 1999; Kircher et al., 1999; Yamaguchi et al., 1999; Mas et al., 2000), which suggests that the interaction between phyA and cryptochrome occurs in the nucleus. PhyA also interacts with the nuclear protein PIF3 (Ni et al., 1998), raising the possibility that phytochromes and cryptochromes may form part of larger signaling complexes in the nucleus.

This requirement for cryptochrome in red light signaling is not apparent in red light-mediated inhibition of hypocotyl elongation. *Arabidopsis cry1* and *cry1 cry2* mutant seedlings show a strong inhibition of hypocotyl elongation in both low and high fluence rates of red light. The strong inhibition of hypocotyl elongation seen in cryptochrome mutant seedlings in red light suggests that the requirement for cry1 in phyA signaling to the clock may be unique to the role of phyA in circadian photoperception.

### Model for Circadian Photoperception in Arabidopsis

The circadian clock is of tremendous importance to plants. Not only does it allow them to prepare for the dawn, but it serves as a timekeeper to measure the length of day, a key determinant of flowering time in many species (Simpson et al., 1999; Devlin and Kay, 2000b). Plants show a plasticity in the recruitment of several different photoreceptors under different light conditions. In light input to the clock, all of the photoreceptors examined are capable of mediating the input under certain conditions. Circadian clocks in nature are synchronized to the day/night cycle on a daily basis by dawn and dusk signals: light pulses in the early morning cause phase advances, whereas light pulses in the evening cause phase delays (Daan and Pittendrigh, 1976; Aschoff, 1979). The shortening of periods by increasing light intensity, seen in our *CAB2::LUC* assay, is believed to be the net effect of phase advances and of delays occurring throughout the assay (Daan and Pittendrigh, 1976; Aschoff, 1979). A deficiency in the action of constant light in shortening periods in the various photoreceptor mutants should, therefore, reflect the involvement of these photoreceptors in entrain-



**Figure 6.** Elements Involved in Light Input to the Circadian Clock in Arabidopsis.

Both red light and blue light act in light input to the clock. The phytochromes phyB, phyD and phyE perceive high-fluence-rate red light signals, whereas the cryptochromes cry1 and cry2 perceive high-fluence-rate blue light signals. Low fluence rates of both red and blue light are perceived by phyA, with cry1 acting in a light-independent manner as a signal transduction component necessary for phyA action.

ment of the clock. This is in agreement with our previous demonstration (Somers et al., 1998) that the deficiency in the perception of low-fluence-rate blue light in the *phyA* mutant affected both the period decrease in constant blue light and the ability to entrain to cycles of blue light and darkness. The involvement of all of the phytochromes and cryptochromes in this phase resetting allows correct entrainment of the circadian clock under a range of light conditions from direct sunlight to dense vegetative shade.

In summary, phyB, phyD, and phyE mediate high-fluence-rate red light input to the clock, and cry1 and cry2 mediate high-fluence-rate blue light input (Figure 6). Perception of both low-fluence-rate red light and low-fluence-rate blue light is mediated by phyA (Figure 6). This research also reveals a new role for plant cryptochrome as a component in the light signal transduction downstream of phytochrome (Figure 6). Although this light-independent role of cryptochrome appears to be limited to light input to the clock, it may, nonetheless, provide some more general clues in the search toward determining the mechanism of action of cryptochromes. Further research is also required to determine the nature of the central oscillator mechanism itself in plants. However, the characteristics of the circadian rhythm observed in the *cry1 cry2* double mutant of *Arabidopsis* make clear that the mechanism of the circadian clock involves quite different molecules in plants and mammals.

### METHODS

#### Generation of Mutant Lines

Wild type, *CAB2::LUC*-expressing lines, and *CAB2::LUC*-expressing lines that were homozygous for *phyA-201*, *phyB-1*, *cry1* (*hy4-2.23M*), or *cry2-1* (*Arabidopsis thaliana* Columbia-4 ecotype) were those described previously (Somers et al., 1998). *phyA phyB* and *phyA cry1*

double mutant lines expressing *CAB2::LUC* were generated by crossing the respective monogenic mutants above and selecting for F<sub>2</sub> plants homozygous for each mutation, using long hypocotyl in far-red light to screen for *phyA*, long hypocotyl in red light to select for *phyB*, and polymerase chain reaction to screen for *cry1* according to the method described by Neff and Chory (1998). *cry1 cry2* double mutants in the Landsberg *erecta* ecotype were selected from a cross of *cry1* expressing *CAB2::LUC* with *cry2 (fha1-1)* by using polymerase chain reaction to select for *cry1* (as above) and *cry2* (with primers provided by D. Weigel, Salk Institute, La Jolla, CA; forward, 5'-GGTTTATCCTGGAAGAGCCCTCAAGATG-3'; and reverse, 5'-CAAGATCGCTGAAATCGTGTGT-3'), followed by digestion with BslI. This yielded fragments of 108 and 21 bp from the wild-type *CRY2* gene and a single 129-bp fragment from the mutant *cry2 (fha1-1)* allele.

### Light Sources

Broadband red light was obtained by filtering output from Sylvania (Danvers, MA) 20-W F20T12/2364 SR5965 red fluorescent tubes through one layer of medium red Roscolene plastic wrap No. 823 (Rosco, Stamford, CT). Broadband blue light was obtained by filtering output from Interelectric Corp. (Warren, PA) Biliblue 20-W F20T12/BBY fluorescent tubes through blue Plexiglas No. 2424 (Commercial Plastics, San Diego, CA). White light was provided by Philips (New York, NY) cool white 20-W F20T12/CW tubes. All light measurements were made with an LI-189 quantum radiometer (Li-Cor, Lincoln, NE).

### Measurement of Period Length

Seeds of wild-type and photoreceptor mutant lines expressing a *CAB2::LUC* reporter construct (Millar et al., 1992) were sterilized and plated on solid Murashige and Skoog medium (Sigma) with 3% sucrose and kanamycin sulfate (50  $\mu\text{g mL}^{-1}$ ). The seeds were stratified at 4°C in the dark for 4 days and then germinated and grown in 12-hr-white-light/12-hr-dark cycles for 6 days. Plastic supporting collars were placed over the seedlings (1.5-cm lengths of clear plastic drinking straws were sterilized by soaking in 70% ethanol; they were then dried and inserted into the agar medium) before transfer to constant red light (600 to 700 nm), blue light (400 to 500 nm), or white light at the intensities indicated. The rhythm of bioluminescence, representing *CAB2* transcription, was monitored as described previously (Millar et al., 1995), and the period length was calculated by fitting a cosine wave function to the time series for each seedling (Millar et al., 1995; Somers et al., 1998). Each data point represents the mean ( $\pm$ SE) for five to 18 seedlings. Each plot is representative of two to four independent experiments.

### Measurement of Hypocotyl Length

Seeds of wild-type and photoreceptor-mutant lines were plated on solid Murashige and Skoog medium (Sigma), stratified at 4°C in the dark for 4 days, and then given a 2-hr white light pulse (50  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) at 22°C before being transferred to darkness, low-fluence-rate (0.3  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ), or high-fluence-rate (30  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ) red light. Hypocotyl length was measured after 3 days of treatments by using Scion Image software (Scion, Frederick, MD) to analyze digital images of seedlings laid out flat on agar plates. Data represent the mean ( $\pm$ SE) of 30 seedlings for each treatment.

### RNA Extraction and Gel Blotting

RNA extraction and detection of *CCR2* message by RNA gel blotting were performed exactly as described by Somers et al. (2000).

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# Cryptochromes Are Required for Phytochrome Signaling to the Circadian Clock but Not for Rhythmicity

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