Phytochromes and Cryptochromes in the Entrainment of the *Arabidopsis* Circadian Clock

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Circadian clocks are synchronized by environmental cues such as light. Photoreceptor-deficient *Arabidopsis thaliana* mutants were used to measure the effect of light fluence rate on circadian period in plants. Phytochrome B is the primary high-intensity red light photoreceptor for circadian control, and phytochrome A acts under low-intensity red light. Cryptochrome 1 and phytochrome A both act to transmit low-fluence blue light to the clock. Cryptochrome 1 mediates high-intensity blue light signals for period length control. The presence of cryptochromes in both plants and animals suggests that circadian input pathways have been conserved throughout evolution.

In many organisms, an endogenous, self-sustaining oscillator maintains rhythms of about 24 hours to control a wide variety of biological processes (*1, 2*). To function as a circadian clock, the endogenous oscillator must be entrained to the daily light and temperature cycles of the external environment. However, only a few components are known that connect the endogenous oscillator to the environment (*2, 3*), and no unequivocal connection between a specific photoreceptor and the entrainment of the circadian clock by light has been made in any organism to date (*4*).

Increasing light intensity often tends to lengthen period in nocturnal organisms and to shorten period in diurnal organisms, including plants (*5*). At least two types of unidentified photoreceptor systems mediate light input in the algae *Gonyaulax* and *Chlamydomonas* (*6*). To address this question in higher plants, we crossed *Arabidopsis* red light (RL) and blue light (BL) photoreceptor mutants with transgenic plants containing the firefly luciferase gene (*luc*) under the control of the clock-responsive *cab2* promoter (*7*).

Plant photoreceptors absorb primarily in RL [600 to 700 nm wavelength; the phytochromes (phy)] and BL [400 to 500 nm; the cryptochromes (cry) and *NPH1*] (*8*). Limiting RL input, either by reducing fluence rate or photoreceptor abundance [(*9*); supplementary figure 1, available at www.sciencemag.org/feature/data/985395.shl] slows the pace of the oscillator, suggesting phytochromes are the likely mediators of RL signaling to the clock. To identify which of the five phytochrome species (*PHYA-E*) (*10*) control RL input to the clock in *Arabidopsis*, we tested null mutant alleles of two phytochromes, phyA and phyB, for their effects on the free-running rhythm in constant RL.

A phyA deficiency affected period length of the clock in RL only in dim red light (<1.0 μmol m^{-2} s^{-1}) (Fig. 1A). These results support previous research showing that phyA mediates developmental responses to long-term, high-irradiance far-red (FR)–enriched light or to pulses of red and FR light (*11*). One exception is in the coupling of phyA to the photoperiodic control of flowering. Both in pea and *Arabidopsis*, white-light–grown phyA mutants have an altered flowering time, suggesting an interaction between phyA and circadian timing (*12*). Our results indicate that this is not through a direct effect of phyA-mediated input to the clock, but more likely the result of clock-gated control of an independent phyA signaling pathway.

In contrast, the phyB-deficient mutant mediates high-fluence RL input to the circadian clock, showing a 1.5- to 2-hour lengthening of period, relative to the wild type, at fluence rates >5.0 μmol m^{-2} s^{-1} (Fig. 1B). Overexpression (15-fold) of phyB (*13*) shortened period length by 1 to 3 hours, relative to the wild type, dependent on the fluence rate [(*14*); supplementary figure 2, available at www.sciencemag.org/feature/data/985395.shl]. It is likely that other phytochromes (phy C, D, or E, or a combination of these) are also involved, because the free-running period in the *phyB* mutant was still shorter at the higher fluence range (>5μmol m^{-2} s^{-1}) than in the phytochrome-deficient *hy1-6* line (*14, 9*), and it continues to shorten up to 200 μmol m^{-2} s^{-1} (Fig. 1B). Tests using single and multiple *phy* mutant combinations will be necessary to fully describe this signaling network (*15*).

Because phytochrome also absorbs BL, we examined the BL fluence rate–response curve of the *phyA* and *phyB* mutants. At fluence rates below 3 to 5 μmol m^{-2} s^{-1}, the absence of phyA lengthened the free-running period by up to 3 hours, relative to the wild type (Fig. 1C), demonstrating that phyA is required for period control in low-fluence BL, and an additional photoreceptor is required to mediate the full range of response to BL. A deficiency in phyB had no period effect in BL (Fig. 1D).

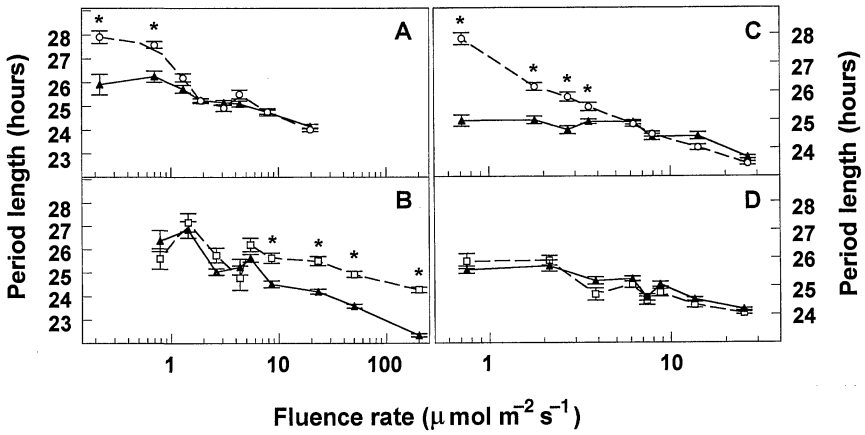

Fig. 1. Effect of RL fluence rate on free-running period length of *cab2::luciferase (cab2::luc)* expression in (**A**) *phyA-201* and (**B**) *phyB-1* mutant seedlings. Effect of BL fluence rate on free-running period length of *cab2::luc* expression in (**C**) *phyA-201* and (**D**) *phyB-1* mutant seedlings (*23*). ○, *phyA-201*; □, *phyB-1*; ▲, Laer wild type. Plants were germinated and grown in cycles of 12 hours white fluorescent light (50 to 60 μmol m^{-2} s^{-1}) and 12 hours of dark for 6 days, then transferred for >110 hours to continuous red (600 to 700 nm) or blue (400 to 500 nm) light at the fluence rates indicated. Mean period length estimates were obtained by fitting a modified cosine wave function to the time series of each seedling (*9, 24*). Luminescence measurements of *cab2::luc* expression were described previously (*9*). Error bars are ± SEM (n = 7 to 18). Asterisk, $P < 0.01$ (Student's two-tail heteroscedastic t test). Similar results were obtained in 2 to 11 independent experiments.

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To further establish the role of phyA in the BL entrainment pathway, a second type of assay was performed. Seedlings grown in white-light/dark (LD) cycles (12 hours/12 hours; T = 24 hours) were shifted to one of eight different BL/dark cycles (10 hours/10 hours; T = 20 hours), spanning a >30-fold range of BL fluence rates. We reasoned that at high fluence rates, the *phyA* mutant would entrain to the 20-hour T cycle, but at the lowest intensities, BL input to the clock would be impaired and the plants would show a 24-hour rhythm, in phase with the original white-light entrainment protocol.

At the highest fluence rate (27 μmol m^{-2} s^{-1}), both strains entrained rapidly (Fig. 2A). The wild type showed similar results at a much lower fluence rate (2 μmol m^{-2} s^{-1}), whereas the *phyA* mutant required at least three cycles to become stably entrained (Fig. 2B). In the mutant, peak luminescence after hour 20 came 4 hours later than the wild type (Fig. 2B; arrowhead), and there was no response to the lights coming on at hour 40. Poor entrainment also occurred at lower fluence rates (0.8 μmol m^{-2} s^{-1}), but at greater than ~4 μmol m^{-2} s^{-1}, the *phyA* mutant entrained as rapidly as the wild type [(*14*); supplementary figure 3, available at www.sciencemag.org/feature/data/985395.shl]. These results match the range of fluence rates over which a phyA deficiency causes a lengthening of period in the fluence rate–response curve (Fig. 1C). The similarity of the fluence rate range over which phyA affects entrainment under these two conditions supports the notion that light signaling to the clock in continuous illumination and light/dark cycles share some similar properties.

The cryptochromes (cry1 and cry2) share similarity with photolyases and are present in both plants and animals (*16*, *17*). Both *Arabidopsis* cryptochromes have been linked to blue-light–dependent processes in plant development, including flowering time (*16*). Overexpression of cry1 shortened period by 1 to 1.5 hours in both high-fluence white (50 μmol m^{-2} s^{-1}) and blue (18 μmol m^{-2} s^{-1}) light (*14*). Conversely, loss of cry1 resulted in period lengthening over two different ranges of BL fluence rates (>10 μmol m^{-2} s^{-1}; <3 μmol m^{-2} s^{-1}) (Fig. 3A). Between these two intensity ranges, the loss of cry1 was inconsequential, suggesting that other BL receptors mediate signaling in this region. The absence of cry2 caused minimal period lengthening in the high fluence rate range (>10 μmol m^{-2} s^{-1}) (Fig. 3B). Over a narrow intensity range (Fig. 3B; 3 to 4 μmol m^{-2} s^{-1}), loss of cry2 showed a slight but reproducible period shortening.

Cry1 and cry2 may act additively and redundantly, and only the double mutant will reveal the combined effect of their overlapping actions. As well, at fluence rates <3 to 5 μmol m^{-2} s^{-1}, cry1 and phyA deficiencies each cause similar period lengthening, suggesting that the two together are required for normal BL signaling. This is supported by recent yeast two-hybrid interaction studies showing a direct interaction between cry1 and phyA polypeptides (*18*).

When grown in white light, *cry2-1* is day-length-insensitive and late-flowering (*19*). To determine whether this effect on photoperiodic timing correlates with a change in period length, we determined the free-running period of all individuals of an F_2 population segregating for *cry2-1* in white light. Although the population segregated 1:3 (P > 0.10) for long-period:wild-type–period, the 16 *cry2-1* homozygotes were distributed as 1/4 of the individuals (P > 0.50) within each of the two period classes, as expected for single gene segregation independent of period length (Fig. 4). Loss of cry2, therefore, does not correlate with a change in period length when grown in white light, and the slight period lengthening in BL (Fig. 3B) has no effect on flowering time (*19*). The effects of cry2 on photoperiodic timing most likely arise through a circadian-clock gating of cry2-mediated signaling, and not through a direct signaling of the cry2 to the oscillator.

Our evidence for multi-photoreceptor–mediated control of the pace of the circadian clock is in concert with recent physiological and molecular genetic studies of phototransduction in plants. Studies using photoreceptor-specific null mutants in *Arabidopsis* have indicated that the nature of the interactions among phyA, phyB, and cry1 signaling pathways is strongly dependent on light quality and fluence rate (*20*). Similar conclusions can be derived from recent work showing direct molecular interactions between phyA and cry1 (*18*).

For sedentary, light-dependent organisms such as plants, the development of compensatory mechanisms against the potentially disruptive effects of wide changes in the light environment on physiology and development may be essential. By recruiting a diversity of photoreceptors that can cover a wide range of fluence rates and spectral qualities, the plant can ensure that the pace of the circadian oscillator remains little affected. This in turn

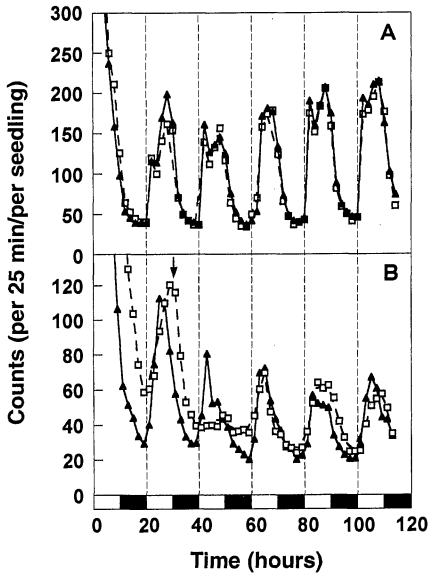

Fig. 2. Re-entrainment of *cab2::luc* expression rhythm to light/dark (10 hour/10 hour) cycles in wild-type and *phyA-201* mutant seedlings. Wild-type (Ler) (▲) and *phyA-201* mutant (□) seedlings were grown in cycles of 12 hours white fluorescent light (50 to 60 μmol m^{-2} s^{-1}) and 12 hours dark for 6 days, then transferred (hour 0) to BL/dark (10 hour/10 hour) cycles at fluence rates of (**A**) 27 μmol m^{-2} s^{-1} or (**B**) 2 μmol m^{-2} s^{-1}. For each trace, n = 18; error bars are omitted for clarity. Open boxes on the time axis indicate BL; filled boxes, darkness. Representative of two independent experiments.

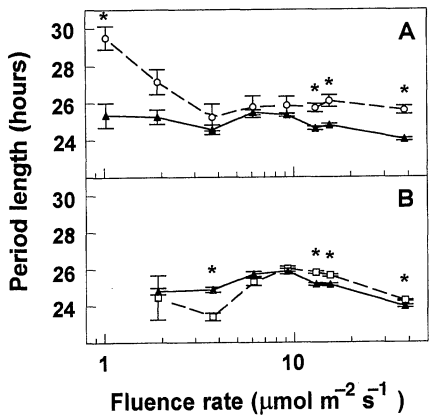

Fig. 3. Effect of BL fluence rate on free-running period length of *cab2::luc* expression in (**A**) *cry1* and (**B**) *cry2* mutant seedlings. ○, *cry1*; □, *cry2*; ▲, Ler or Col-1 wild type. Conditions of entrainment, luminescence measurements and data analysis as in Fig. 1. Error bars are ± SEM (n = 5 to 19). *, P < 0.01 (Student's two-tail heteroscedastic t test). Representative of four independent experiments (*25*).

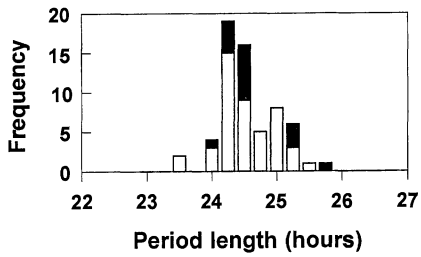

Fig. 4. Period length distribution in a seedling population segregating for *cry2-1*. Entrainment protocol, free-run conditions (50 to 60 μmol m^{-2} s^{-1} white light), luminescence measurements, and period analysis are as in Fig. 1. Period bins are labeled with the upper bound. Dark fill shows *cry2-1* homozygotes (*25*). Representative of two independent experiments.

ensures that circadian-controlled processes maintain the appropriate phase relationship to environmental cues. Photoreceptor diversity and redundancy, therefore, appear to be the key features in the photocontrol of the circadian clock in higher plants.

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23. Individual *cab2::luc*-expressing lines homozygous for *phyA-201* or *phyB-1* were selected from a cross of *phyA-201* (*21*) with 2CAC (C24 ecotype) (9), selfed, and harvested individually (*phyA* F_3 pools), or selected and selfed from a second backcross of 2CAC to *phyB-1* (*21*) (*phyB* F_3 pools). 2CAC/Laer(7x) F_2 seedlings [from the seventh backcross of 2CAC with ecotype Landsberg *erecta* (Laer)] were used as the wild-type control. Homozygous *phyA-201* and *phyB-1* mutants were selected on the basis of hypocotyl length in far-red and red light, respectively. Light sources in this work were described previously (9). Repeat trials used two to three independent F_3 pools.
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Role of Mouse Cryptochrome Blue-Light Photoreceptor in Circadian Photoresponses

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Cryptochromes are photoactive pigments in the eye that have been proposed to function as circadian photopigments. Mice lacking the cryptochrome 2 blue-light photoreceptor gene (*mCry2*) were tested for circadian clock-related functions. The mutant mice had a lower sensitivity to acute light induction of *mPer1* in the suprachiasmatic nucleus (SCN) but exhibited normal circadian oscillations of *mPer1* and *mCry1* messenger RNA in the SCN. Behaviorally, the mutants had an intrinsic circadian period about 1 hour longer than normal and exhibited high-amplitude phase shifts in response to light pulses administered at circadian time 17. These data are consistent with the hypothesis that CRY2 protein modulates circadian responses in mice and suggest that cryptochromes have a role in circadian photoreception in mammals.

Circadian rhythms are oscillations in the biochemical, physiological, and behavioral functions of organisms with a ~24-hour periodicity (*1*). Circadian rhythms are synchronized with light-dark cycles, but the molecular basis of this "photoentrainment" is not known (*2*). Indeed, there is no consensus on the nature of the circadian photoreceptor. Three classes of pigments have been considered as candidates: opsin/retinal-based photopigments (*3*), tetrapyrrole-based heme pigments (*4*), and pterin/flavin-containing cryptochrome blue-light photoreceptors (*5, 6*). Cryptochromes were first identified in plants as structural homologs of the DNA repair enzyme DNA photolyase (*7*), but they lack DNA repair activity (*8*) and are involved in mediating growth (*9*), flowering time (*10*), and phototropism (*11*) in response to blue light. Recently, two human and mouse homologs of the plant cryptochromes were discovered (*5, 12*). Cryptochromes 1 and 2 (CRY1 and CRY2, respectively) lack DNA repair activity (*5*) and are expressed in the mouse retina (*6*), and *mCry1* exhibits circadian oscillations of expression in the SCN (*6*) wherein the central pacemaker of the body resides. These observations led to the proposal that cryptochromes were likely to be photopigments for circadian photoentrainment (*6*).

To test this hypothesis, we created a mouse strain that lacks the predominant form of cryptochrome found in the mouse retina, CRY2, and analyzed its circadian behavior using biochemical and behavioral tests. We generated $Cry2^{+/-}$ heterozygous mutant mice by established methods (*13*) using the targeting construct shown in Fig. 1A. Interbreeding of heterozygotes yielded progeny of wild type:$Cry2^{+/-}$:$Cry2^{-/-}$ at a ratio of 1:2:1 (Fig. 1B). The mutant mice were phys-

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