

# A Constitutively Active Allele of Phytochrome B Maintains Circadian Robustness in the Absence of Light<sup>1[OPEN]</sup>

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The sensitivity of the circadian system to light allows entrainment of the clock, permitting coordination of plant metabolic function and flowering time across seasons. Light affects the circadian system via both photoreceptors, such as phytochromes and cryptochromes, and sugar production by photosynthesis. In the present study, we introduce a constitutively active version of phytochrome B-Y276H (YHB) into both wild-type and phytochrome null backgrounds of *Arabidopsis* (*Arabidopsis thaliana*) to distinguish the effects of photoreceptor signaling on clock function from those of photosynthesis. We find that the YHB mutation is sufficient to phenocopy red light input into the circadian mechanism and to sustain robust rhythms in steady-state mRNA levels even in plants grown without light or exogenous sugars. The pace of the clock is insensitive to light intensity in YHB plants, indicating that light input to the clock is constitutively activated by this allele. Mutation of YHB so that it is retained in the cytoplasm abrogates its effects on clock function, indicating that nuclear localization of phytochrome is necessary for its clock regulatory activity. We also demonstrate a role for phytochrome C as part of the red light sensing network that modulates phytochrome B signaling input into the circadian system. Our findings indicate that phytochrome signaling in the nucleus plays a critical role in sustaining robust clock function under red light, even in the absence of photosynthesis or exogenous sources of energy.

The circadian system has evolved as an endogenous time-keeping mechanism that confines many biochemical and physiological processes to specific parts of the day and allows plants to accurately measure seasonal transitions (for review, see Song et al., 2013; Hsu and Harmer, 2014). To remain synchronized with the regular diurnal cycle, the plant circadian system is exquisitely sensitive to environmental changes in light and temperature (Fankhauser and Staiger, 2002; Jones, 2009). Although specific temperature sensors have remained elusive, light input to the circadian system

occurs primarily via phytochromes, cryptochromes, and the ZEITLUPE family of photosensory F-box proteins (Somers et al., 1998, 2000; Devlin and Kay, 2000; Pudasaini and Zoltowski, 2013).

Phytochromes (phys) are the primary red and far-red light photoreceptors in plants (Bae and Choi, 2008) and consist of a five-member protein family in *Arabidopsis* (*Arabidopsis thaliana*; Clack et al., 1994; Franklin and Quail, 2010). Phys reversibly switch between P<sub>r</sub> and P<sub>fr</sub> forms upon absorption of red or far-red light, respectively (Rockwell et al., 2006), and the ratio of these forms within the cell controls the shade avoidance response and contributes to light perception (Casal, 2013). PhyA is the most divergent phy, with a specialized role as a far-red light sensor (Casal et al., 2014), whereas phyB is the predominant red light sensor in *Arabidopsis* (Whitelam and Devlin, 1997). Although oscillation of circadian transcripts is dampened under constant far-red light, phyA retains photoregulatory control of these and other genes under these conditions (Wenden et al., 2011). By contrast, phyA, phyB, and phyD each appear to contribute to maintenance of circadian rhythms under constant red light (Rc; Somers et al., 1998; Devlin and Kay, 2000). *PhyD* single mutants have a wild-type circadian phenotype but have an additive effect when introgressed into a *phyB* background (Devlin and Kay, 2000). Although recent studies on temperate grasses have established a direct role for phyC in photoperiod sensing (Chen et al., 2014; Woods et al., 2014), phyC has not been formally described as

<sup>1</sup> This work was supported by the National Institutes of Health (grant nos. GM069418 to S.L.H. and GM068552 to J.C.L.), U.S. Department of Agriculture National Institute of Food and Agriculture (Hatch Project CA-D\*-MCB-4126-H to J.C.L.), the Leverhulme Trust (grant no. ECF-2012-358 to M.A.J.), the Royal Society (grant no. RG130746 to M.A.J.), the Oppenheimer Memorial Trust (PhD studentship to S.L.), and the University of Essex (to M.A.J. and S.L.). M.A.J. is a Leverhulme Early Career Fellow.

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M.A.J., W.H., J.C.L., and S.L.H. designed the research; M.A.J., W.H., and S.L. performed the research; M.A.J. and W.H. contributed new tools; M.A.J., W.H., S.L., J.C.L., and S.L.H. analyzed the data; M.A.J., W.H., J.C.L., and S.L.H. wrote the article.

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[www.plantphysiol.org/cgi/doi/10.1104/pp.15.00782](http://www.plantphysiol.org/cgi/doi/10.1104/pp.15.00782)

part of the circadian system in *Arabidopsis*. However, phyC is presumed to act similarly to phyE as a modulator of phyB activity since neither phyC nor phyE is capable of forming the homodimers necessary for signaling activity in *Arabidopsis* (Clack et al., 2009) or in rice (*Oryza sativa*; Xie et al., 2014), and instead, both function as heterodimers with other phys.

The circadian system is typically conceptualized as a core molecular oscillator reset by light and temperature stimuli that regulates the expression of multiple output pathways (Harmer, 2009). Outputs are easily defined as processes under circadian control that do not feed back into the circadian system; however, the distinction between input and core components of the circadian system has become increasingly blurred with our expanding knowledge of the circadian system. For example, phys transduce light signals from the red and far-red portion of the spectrum into the circadian system, but the transcription of this photoreceptor family is concurrently regulated by the clock (Somers et al., 1998; Bognár et al., 1999; Devlin and Kay, 2000; Tóth et al., 2001; Wenden et al., 2011). Despite this complication, the core oscillator is generally considered to consist of a complex web of interacting feedback loops that are sufficient to generate a self-sustaining oscillation of transcripts and proteins (Fogelmark and Troein, 2014; Hsu and Harmer, 2014). Although the relative effect of light on each core circadian component has not been systematically determined, several reports have identified transcripts that are acutely induced by light (Wang and Tobin, 1998; Ito et al., 2003; Locke et al., 2005). CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are morning-phased, light-inducible transcription factors that induce expression of *PSEUDO-RESPONSE REGULATOR9* (*PRR9*; Schaffer et al., 1998; Wang and Tobin, 1998; Farré et al., 2005). *PRR9*, whose transcription is itself light induced (Ito et al., 2003; Jones et al., 2012), represses expression of *CCA1* and *LHY* in partnership with the later-phased *PRR7*, *PRR5*, and *TIMING OF CAB1* (*TOC1*; Matsushika et al., 2000; Nakamichi et al., 2010). Later in the subjective day, *TOC1* and *REVEILLE8* act as negative and positive transcription factors, respectively, that act to reinforce the robust oscillations of circadian genes (Farinas and Más, 2011; Gendron et al., 2012; Huang et al., 2012; Hsu et al., 2013). In addition to acute photoreceptor-mediated effects on the clock, it is well established that increasing fluence rates of light under constant conditions quickens circadian pace in plants, as well as many other species (Aschoff, 1960). For plants, the shorter period length seen at higher fluence rates of light may be due in part to increased photosynthesis, a process that is strongly regulated by phys and other photoreceptors (Haydon et al., 2013). However, *Arabidopsis* seedlings lacking all five phytochromes exhibit a shorter circadian period than the wild type under dim light, despite being deficient in photosynthetic light capture (Strasser et al., 2010; Hu et al., 2013).

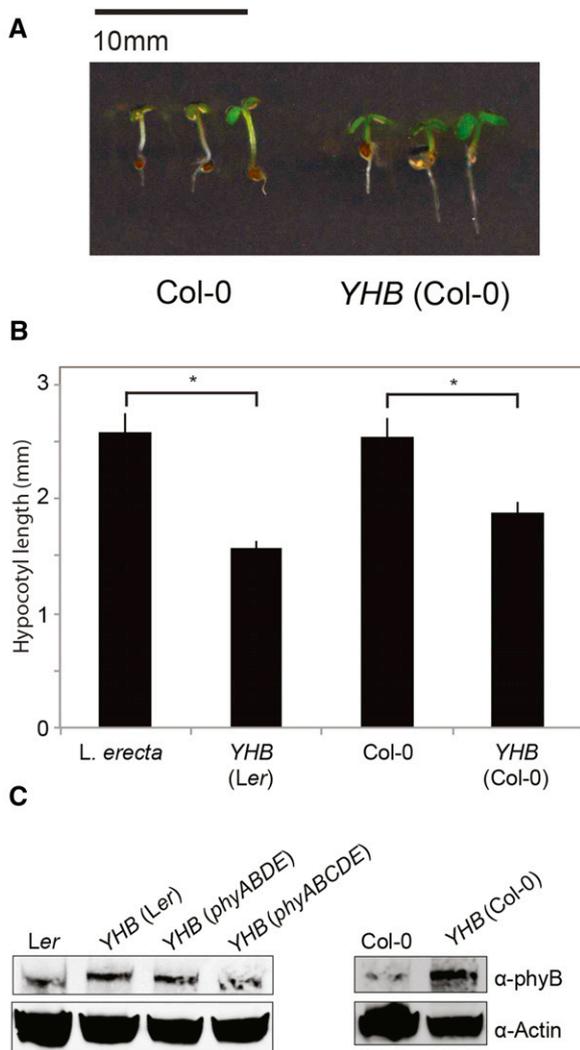
The ability of phyB-E to form homo- and heterodimers introduces complexity into the interpretation of individual phy mutant phenotypes (Sharrock and Clack, 2004; Clack et al., 2009; Hu et al., 2013). In addition, period length in *phyABCDE* mutants is modestly sensitive to different fluence rates of red light (Hu et al., 2013), suggesting that metabolic processes may complicate the assessment of the role of Phys in light input to the circadian clock. To better understand phy-dependent and phy-independent light signaling inputs to the circadian system, we now exploit the *phytochrome B-Y276H* (*YHB*) mutant allele that induces constitutive phyB signaling in the absence of light (Su and Lagarias, 2007; Hu et al., 2009). By introducing the *YHB* allele into the *phyABCDE* background, we resolve the direct roles of red light from those of phyB activation on clock output/function. We demonstrate that nuclear-localized but not cytosolic *YHB* is sufficient to maintain circadian rhythmicity in constant darkness in the absence of endogenous photoreceptor activation or photosynthesis. Our studies also identify a regulatory role for phyC within the circadian system to enhance phyB signaling input under dim red light.

## RESULTS

### *YHB* Sustains Core Clock Transcript Cycling in Light-Grown Plants Transferred to Constant Darkness

Previous work has demonstrated that *YHB* is sufficient to induce photomorphogenesis and to initiate transcriptional cascades that mimic red light-induced phy signaling in the absence of light (Su and Lagarias, 2007; Hu et al., 2009). The circadian system is exquisitely sensitive to changes in day length and light intensity (Salomé et al., 2008; Jones, 2009), so we were curious whether the *YHB* allele would mimic phyB-mediated light input into the circadian system. We therefore crossed the transgenic genomic *YHB* allele into *Arabidopsis* plants carrying a clock-regulated bioluminescent reporter. Previous screens have used a *COLD*, *CIRCADIAN RHYTHM AND RNA BINDING2* (*CCR2*::*LUCIFERASE* (*LUC*) reporter (Columbia accession [Col]) to measure circadian rhythms in the dark (Martin-Tryon et al., 2007), and so we generated Col plants containing both the *YHB* allele and the *CCR2*::*LUC* reporter in addition to introducing a *CCA1*::*LUC2* reporter into existing *YHB* (*Landsberg erecta* [*Ler*]) lines (Hu et al., 2009). Introduction of the *YHB* allele confers shortened hypocotyls and expanded cotyledons in *Ler* seedlings grown in darkness or in constant light (Su and Lagarias, 2007; Hu et al., 2009). Similar short hypocotyls were observed in our newly generated *YHB* (Col) lines when compared with wild-type Col controls grown under white light ( $P < 0.05$ ; Fig. 1, A and B). *YHB* was moderately overexpressed compared with endogenous phyB in these lines (Fig. 1C).

We initially tested whether constitutively active *YHB* protein would be sufficient to maintain robust luciferase rhythms in light/dark (L/D)-entrained plants transferred to constant darkness (Fig. 2, A–D). Assessments of circadian rhythms have historically used Suc



**Figure 1.** Characterization of an additional YHB allele. A, Morphology of seedlings grown under  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles. Wild-type (Col-0) and Col-0 seedlings transformed with a *PHYB::PHYB-Y276H* construct (*YHB*) were grown on Murashige and Skoog (MS) medium for 6 d. B, Quantification of hypocotyl lengths of Col-0, *YHB* (Col-0), *Ler*, and *YHB* (*Ler*) seedlings grown as described in (A). Previously described *Ler* seedlings similarly transformed with *YHB* are presented for comparison (Su and Lagarias, 2007). SEM is presented,  $n > 20$ . \*, Significant difference for the indicated comparison ( $P < 0.001$ , Student's *t* test). C, Immunoblot analysis of phyB/*YHB* protein levels showing relative phyB accumulation in Col-0 and *YHB* (Col-0) seedlings. *Ler* wild type and various transgenic plants harboring the *Ler* *YHB* transgenic allele derived from *YHB/phyA201phyB-5* line #5 (Su and Lagarias, 2007) are presented for comparison.

as a media supplement to enhance bioluminescence in transgenic plants (Millar et al., 1992), although recent work has demonstrated that this exogenous Suc can itself act as an entrainment signal (Dalchau et al., 2011; Haydon et al., 2013). We consequently compared luciferase activity in our *YHB* lines in either the presence or absence of exogenous Suc to facilitate comparison with historical and more recent data sets. We observed that rhythms of *CCR2*-driven luciferase activity in *YHB*

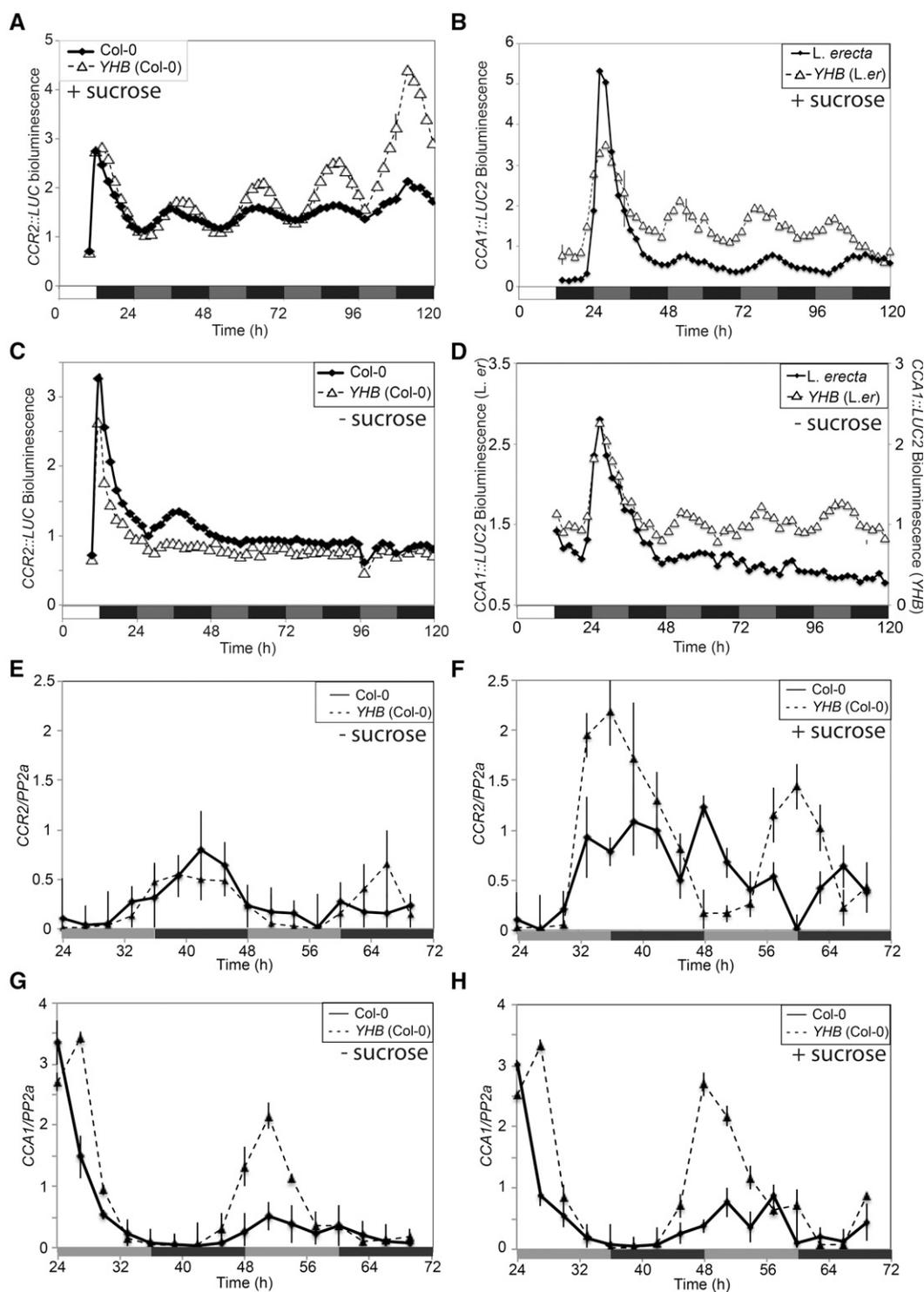
seedlings grown with Suc had increased amplitude throughout the experiment compared with wild-type controls ( $P < 0.001$ ; Fig. 2A), although there was no significant difference in period length between the two populations ( $\tau = 25.4 \pm 0.21$  and  $24.93 \pm 0.08$  for wild-type and *YHB*, respectively;  $P = 0.1$ ). *CCA1*-driven luciferase activity was similarly increased in *YHB* (*Ler*) lines in the presence of Suc (Fig. 2B). In the absence of Suc, we observed that rhythmic bioluminescence in both Col wild-type and *YHB* seedlings containing a *CCR2::LUC* reporter dampened considerably and became arrhythmic following 1 d of constant darkness (Fig. 2C). By contrast, robust circadian rhythms were retained in *YHB* *CCA1::LUC2* lines grown in the absence of Suc ( $\tau = 26.0 \pm 0.23$ ; Fig. 2D). Since rhythmic luminescence activity was not observed from the *CCA1::LUC2* reporter control in control plants in the absence of Suc (Fig. 2D), *YHB* helps maintain robust *CCA1::LUC2* cycling in darkness. Taken together, these studies show that *YHB* enhances cycling amplitudes of both clock output (*CCR2*) and clock gene (*CCA1*) reporters and sustains rhythmic *CCA1*-regulated luciferase activity in the absence of light and Suc.

To better understand the role of exogenous Suc in the maintenance of clock-regulated gene expression in darkness, we examined the steady-state levels of *CCR2* and *CCA1* transcripts in both *YHB* (Col) and wild-type control lines (Fig. 2, E–H). In the absence of Suc, *CCR2* transcript accumulation in the wild type mirrored the luciferase activity data, with one rhythmic peak of transcript accumulation before dampening toward arrhythmia (Fig. 2E). Rhythms were more robust in *YHB* plants grown without Suc, exhibiting two obvious peaks of transcript abundance. Similarly, the abundance of *CCA1* transcripts dampened very quickly in wild-type plants in constant darkness, regardless of the presence of exogenous Suc, whereas rhythmicity was retained in *YHB*-expressing lines (Fig. 2, G and H).

We next performed qRT-PCR analysis of additional core clock transcripts in dark-adapting Col wild-type and *YHB* plants. Following L/D entrainment and transfer to constant darkness at Zeitgeber Time (ZT) 12, we assessed transcript levels of several core circadian clock genes (Hsu and Harmer, 2014). The presence of *YHB* was sufficient to maintain rhythms in transcript levels of the morning-phased genes *CCA1* and *PRR9* as well as the evening-phased genes *GIGANTEA* (*GI*), *TOC1*, and *EARLY FLOWERING4* (*ELF4*; Figs. 2G and 3). In all cases, transcript accumulation rhythms dampened more significantly in wild-type seedlings than in *YHB* seedlings. These results indicate that, independent of the presence of Suc, *YHB* acts to sustain rhythmic expression of core clock transcripts in constant darkness, a phenomenon not seen in wild-type seedlings.

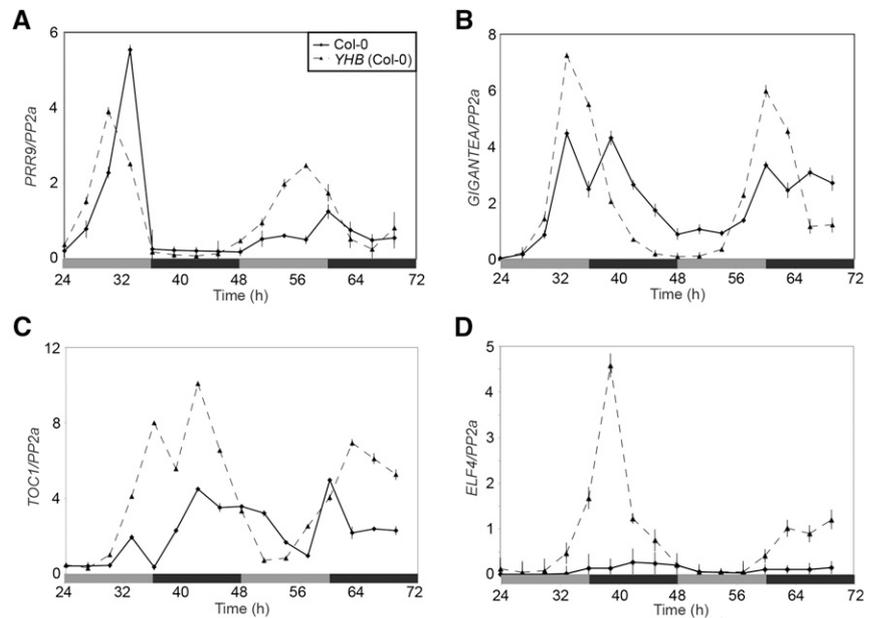
#### Circadian Period of *YHB* Plants Is Insensitive to Fluence Rate

We next examined *YHB* influence on circadian rhythms under a range of intensities of Rc. Following an



**Figure 2.** Effect of YHB on bioluminescence rhythms in constant darkness. A and C, Bioluminescence of Col-0 (solid) and *YHB* (dashed) seedlings containing a *CCR2::LUC* reporter grown on MS medium with (A) or without (C) exogenous Suc;  $n > 9$ . B and D, Bioluminescence of *L. erecta* (solid) and *YHB* (*L. er*; dashed) seedlings containing a *CCA1::LUC2* reporter grown on MS medium with (B) or without (D) exogenous Suc;  $n > 10$ . *YHB* (*L. er*) is presented on a secondary axis in D for clarity. E and F, Abundance of *CCR2* transcripts as measured by quantitative reverse transcription (qRT)-PCR after transfer of seedlings to constant darkness. Plants were grown with (F) or without (E) exogenous Suc as described in A and C. G and H, Abundance of *CCA1* transcripts as measured by qRT-PCR after transfer of seedlings to constant darkness. Plants were grown with (H) or without (G) exogenous Suc as described in A and C. Plants were entrained under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles for 6 d and then transferred to constant

**Figure 3.** *YHB* sustains core clock transcript cycling in L/D entrained plants transferred to constant darkness. Abundance of circadian transcripts as measured by qRT-PCR after transfer of seedlings (grown on Suc-free medium) to constant darkness. Levels of *PRR9* (A), *GI* (B), *TOC1* (C), and *ELF4* (D) mRNA were assessed. Plants were grown on 0.5× MS medium and entrained to 12:12 L/D cycles with 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light for 10 d before transfer to constant darkness at ZT12. mRNA levels for each gene were normalized to *PP2a*; SEM is shown. Gray bars indicate subjective day, whereas black bars indicate subjective night.



entrainment period, wild-type, *YHB*, and *phyB-9* seedlings grown on Suc-free medium were released into Rc, with circadian period and amplitude measured via activity of the *CCR2::LUC* reporter (Fig. 4). Similar to previous reports (Somers et al., 1998; Palágyi et al., 2010), the circadian period of wild-type plants shortened from  $25.1 \pm 0.33$  h to  $22.9 \pm 0.13$  h as the fluence rate increased from 12 to 184  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 4A). *PhyB-9* seedlings exhibited a similar, albeit more exaggerated response over the fluence rates tested, as was reported previously (Fig. 4A; Somers et al., 1998; Palágyi et al., 2010). By contrast, *YHB* seedlings were essentially unresponsive to increasing fluence rates of Rc (up to 184  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), with period length remaining approximately 23.5 h at all fluence rates tested (Fig. 4A). This unresponsiveness resulted in the greatest period difference between *YHB* and the wild type at the lowest fluence rate of Rc (12  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) tested, where a approximately 1.5-h shorter period was observed in the transgenic plant (Fig. 4A). By contrast, under high-intensity Rc, the period lengths of all genotypes were nearly identical. Taken together, these results indicate that period length in *YHB* plants is nearly insensitive to increasing fluence rates of red light.

Differences in the amplitude of bioluminescence rhythms were also detected for *YHB* and wild-type plants after transfer to high fluence rates of Rc. Although similar for all genotypes under 12  $\mu\text{mol m}^{-2} \text{s}^{-1}$  Rc, the amplitude of bioluminescence was greatly enhanced in the wild type with increasing fluence rate of Rc, whereas the responsiveness of the *YHB* plants was

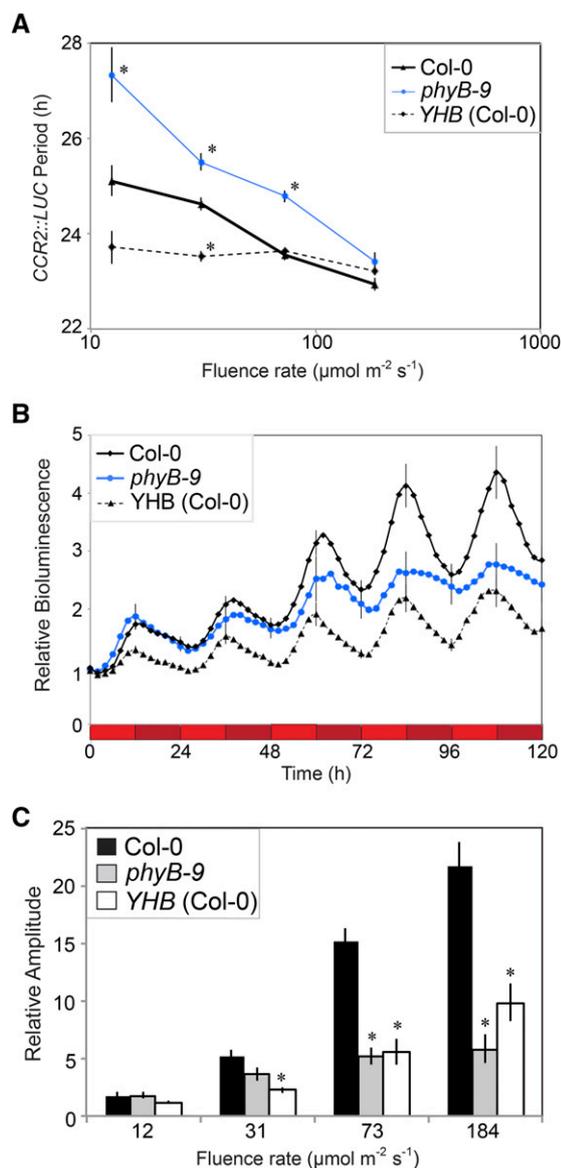
reduced. Indeed, the discrepancy between the amplitude of these genotypes was most pronounced at the highest fluence rates examined, where *YHB* seedlings were half as bright as wild-type controls (Fig. 4, B and C). Similar to the period phenotype, these results indicate that clock amplitude in *YHB* plants is less responsive to increasing fluence rates of Rc than in the wild type.

#### ***YHB* Influences Clock Gene Expression in the Absence of Other Phys**

Type II phys (phyB-E) form homo- and heterodimers that complicate interpretation of phenotypes of loss-of-function *phy* mutants and of gain-of-function *YHB* transgenics (Sharrock and Clack, 2004; Clack et al., 2009; Hu et al., 2009). The additive circadian defect of *phyABD* mutants compared with *phyAB* has been assumed to indicate an ability of phyD to provide light input into the circadian system in the absence of phyB (Devlin and Kay, 2000). However, the loss of phyD potentially also alters the amount of phyC-phyE heterodimers in the two genotypes, providing an alternative explanation for their distinct phenotypes. Similarly, introduction of the *YHB* allele would alter the amounts of the homo- and heterodimeric species of endogenous phyB-E proteins. To better understand how *YHB* and by extension phyB influence the circadian system, we introduced the *YHB* allele into the recently isolated *phyABCDE* quintuple mutant (Hu et al., 2013). In contrast to the photomorphogenesis-challenged phenotypes

**Figure 2.** (Continued.)

darkness at ZT12 on day 6. Gray bars indicate subjective day, whereas black bars indicate subjective night. *CCR2* and *CCA1* mRNA levels were normalized to *PP2a*. SEM is shown.



**Figure 4.** YHB suppresses the clock's response to increasing fluence rates of red light. **A**, Fluence rate response curve to measure free-running circadian period under red light. *phyB-9* (blue) and *YHB-9* (dashed) alleles were crossed into a Col-0 background (solid line) carrying a *CCR2::LUC* luciferase reporter. Homozygous lines were grown under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles on  $0.5 \times$  MS medium for 6 d before transfer to Rc at the indicated fluence rate on day 6. **B**, Example of circadian rhythms observed in **A**. Seedlings were entrained as described in **A** before being transferred to  $184 \mu\text{mol m}^{-2} \text{s}^{-1}$  Rc. Shaded red bars indicate subjective night. **C**, Amplitude of luciferase rhythms reported in **A**. SEM is shown. \*, Significant difference compared with the wild type, Bonferroni adjusted Student's *t* test.

of the *phyABCDE* parental line under 12:12 L/D cycles, *YHB(phyABCDE)* plants looked similar to wild-type seedlings with short hypocotyls and expanded cotyledons (Fig. 5).

To further explore the effect of YHB in the absence of other phys, we assessed the accumulation of core clock

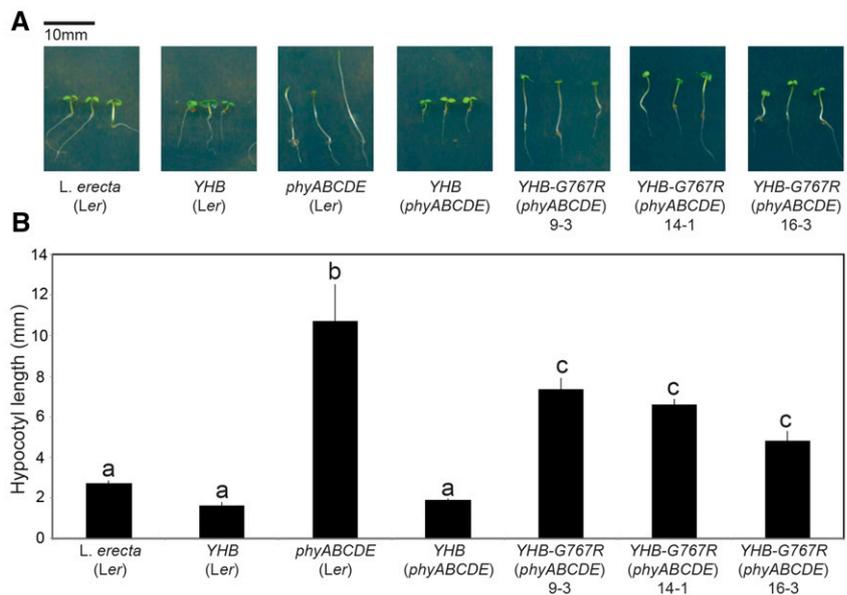
gene transcripts in the *phyABCDE* mutant background using qRT-PCR. The *phyABCDE* mutant was generated in the *Ler* background; hence, we used a previously reported *YHB* line [*YHB(Ler)*; Su and Lagarias, 2007; Hu et al., 2009] as a control. As observed for *YHB(Ler)* plants, *YHB(phyABCDE)* seedlings transferred to constant darkness displayed robustly rhythmic *CCA1* accumulation, a response that is strongly damped in the *Ler* wild type (Fig. 6A). *YHB(phyABCDE)* also sustained rhythmic expression of *PRR9*, *GI*, and *ELF4* transcripts in prolonged darkness (Fig. 6, B–D). All of these clock genes displayed dampened oscillations in the dark-adapting *Ler* wild type, similar to our results in the Col accession (Figs. 2 and 3). These data indicate that endogenous phys are not required for YHB-mediated maintenance of robust circadian rhythms in darkness.

#### Cytosolic YHB Has Little Effect on Clock Gene Expression or Circadian Pace

In contrast to endogenous phy, YHB does not require light activation to migrate from the cytoplasm to the nucleus and is instead constitutively targeted to the nucleus (Su and Lagarias, 2007). In the nucleus, YHB acts similarly to the  $P_{fr}$  form of phyB by binding PHYTOCHROME INTERACTING FACTOR (PIF) basic Helix-Loop-Helix (bHLH) transcription factors and targeting them for degradation (for review, see Bae and Choi, 2008). More recently, signaling roles for  $P_{fr}$  in the cytoplasm have been reported (Paik et al., 2012; Hughes, 2013). To evaluate the contribution of cytoplasmic  $P_{fr}$  signaling into the circadian system, we introduced the G767R mutation into the *YHB* allele (*YHB-G767R*). Phys containing the G767R mutation are retained in the cytoplasm (Wagner and Quail, 1995; Ni et al., 1999; Matsushita et al., 2003). This has been attributed to the inability of the G767R mutant to interact with PIF3 and then be imported into the nucleus (Pfeiffer et al., 2012). Surprisingly, the double phy mutant partially complemented the *phyABCDE* null mutant: light-grown *YHB-G767R(phyABCDE)* lines exhibited expanded cotyledons and shorter hypocotyls compared with the parental *phyABCDE* seedlings (Fig. 5). However, these results contrast with the strong hyperactivity of YHB in both null and wild-type backgrounds (Fig. 5). Thus, it is clear that the G757R mutation largely suppresses the gain-of-function activity of YHB, presumably by retaining it in the cytosol.

To assess the role of cytoplasmic *YHB-G767R* within the circadian system, we used qRT-PCR to assess its effects on transcript levels of genes with poor cycling in wild-type plants maintained in constant darkness in the absence of exogenous Suc (Fig. 6, A–D). Whereas *YHB*-expressing plants demonstrated robust rhythms in steady-state mRNA levels, dark-adapting *YHB-G767R(phyABCDE)* lines grown in the absence of Suc showed rapidly damping rhythms very similar to those observed in dark-adapting wild-type *Ler*.

**Figure 5.** Morphology of seedlings transformed with YHB and YHB-G767R alleles. A, Seedlings were grown on  $0.5 \times$  MS medium without Suc for 6 d under  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles. B, Quantification of hypocotyl lengths of seedlings shown in A. SEM is presented,  $n > 20$ . Letters indicate significantly different populations ( $P < 0.001$ , Tukey's honestly significant difference test).



We next examined the effects of activated phy containing the G767R mutation on circadian pace. The YHB or YHB-G767R transgenes were crossed into *phyABCDE* mutants expressing the *CCA1::LUC2* reporter, and rhythms in bioluminescence activity were assessed in dark-adapting plants grown in the presence of exogenous Suc. Whereas *YHB(phyABCDE)* plants had a shorter period of  $23.68 \pm 0.08$  h compared with the parental *phyABCDE* ( $26.57 \pm 0.48$  h), the period length of *YHB-G767R(phyABCDE)* plants was indistinguishable from the control ( $27.04 \pm 0.37$  h). These data indicate that both the shortening of circadian period by YHB and its enhancement of rhythms in transcript abundance are dependent upon its nuclear localization.

#### PhyC Modulates Light Input to the Circadian System

PhyC protein does not accumulate in *phyABDE* seedlings (which therefore phenocopy *phyABCDE* plants; Hu et al., 2013), and thus Arabidopsis phyC function depends upon other phys. Since phyC forms heterodimers with phyB or phyD (Clack et al., 2009), we were curious whether the presence of phyC was able to alter the activity of YHB. We therefore introduced the YHB allele into a *phyABDE* background to compare the clock phenotype of these *YHB(phyABDE)* seedlings with that of *YHB(phyABCDE)* plants (Fig. 7, A–C). In these two dark-adapting lines grown in the presence of Suc, the rhythms of *CCA1::LUC2* expression were indistinguishable (Fig. 7A), with periods of  $22.51 \pm 0.24$  and  $23.13 \pm 0.54$  h in *YHB(phyABDE)* and *YHB(phyABCDE)*, respectively. Similarly, in the absence of supplemental Suc, no significant difference in the accumulation of the clock gene transcripts *CCA1* (Fig. 7B), *PRR9*, *GI*, or *TOC1* (Supplemental Fig. S1) was detected between these two lines. Nevertheless, *YHB(phyABDE)* seedlings did exhibit a significantly shorter period than *YHB*

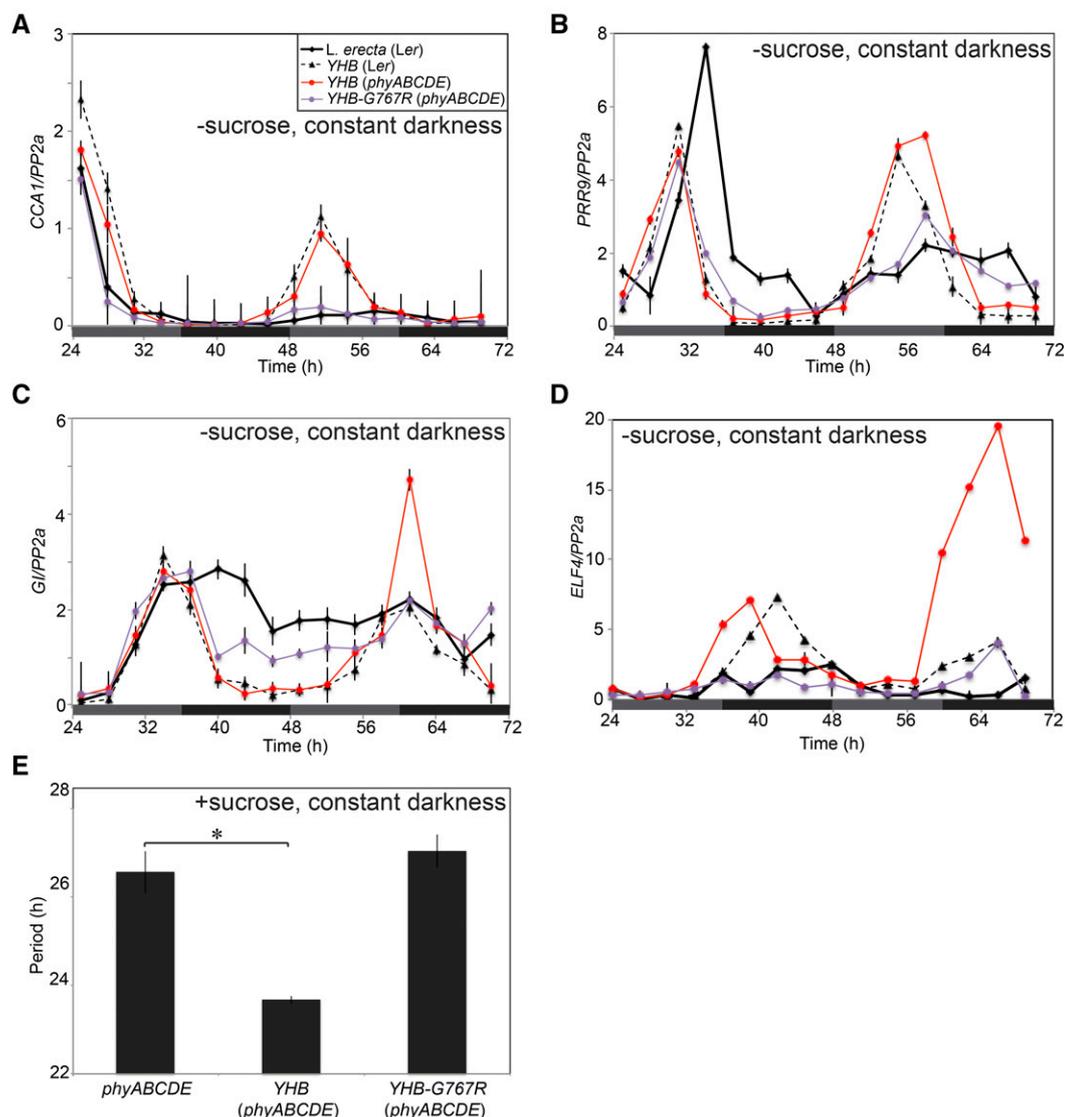
(*phyABCDE*) under dim red light in the absence of Suc ( $\tau = 25.18 \pm 0.42$  and  $27.26 \pm 0.38$ , respectively;  $P = 0.02$ ; Fig. 7C), suggesting that phyC can enhance YHB signaling into the circadian system.

*PhyC* mutants have hypocotyl growth defects (Franklin et al., 2003; Monte et al., 2003), implying an important role of phyC in modulating the activity of other phys (Franklin et al., 2003; Monte et al., 2003; Hu et al., 2013). Our data show that phyC influences the activity of YHB under Rc (Fig. 7C), supporting the hypothesis that phyC acts as a light input into the circadian system. To more directly test this hypothesis, we introduced a *CCA1::LUC2* reporter into *phyC-2* (Monte et al., 2003) and *phyC-4*, two independent T-DNA insertion lines in the Col accession (Fig. 7D). Both *phyC-2* and *phyC-4* mutants had a circadian period approximately 1.5 h longer than wild-type controls under Rc (Fig. 6, D and E), although the amplitude of these rhythms appeared unaffected (Fig. 7D). Similar results were obtained from multiple independent T2 lines transformed with the *CCA1::LUC2* reporter (Supplemental Fig. S2). Both *phyC-2* and *phyC-4* seedlings exhibited longer circadian periods than the wild type across a broad range of fluence rates (Fig. 7F). Such data led us to conclude that phyC modulates red light input into the circadian system in a manner similar to that of phyB (Devlin and Kay, 2000).

## DISCUSSION AND CONCLUSION

### YHB Mimics Continuous Light Input into the Circadian System in Darkness

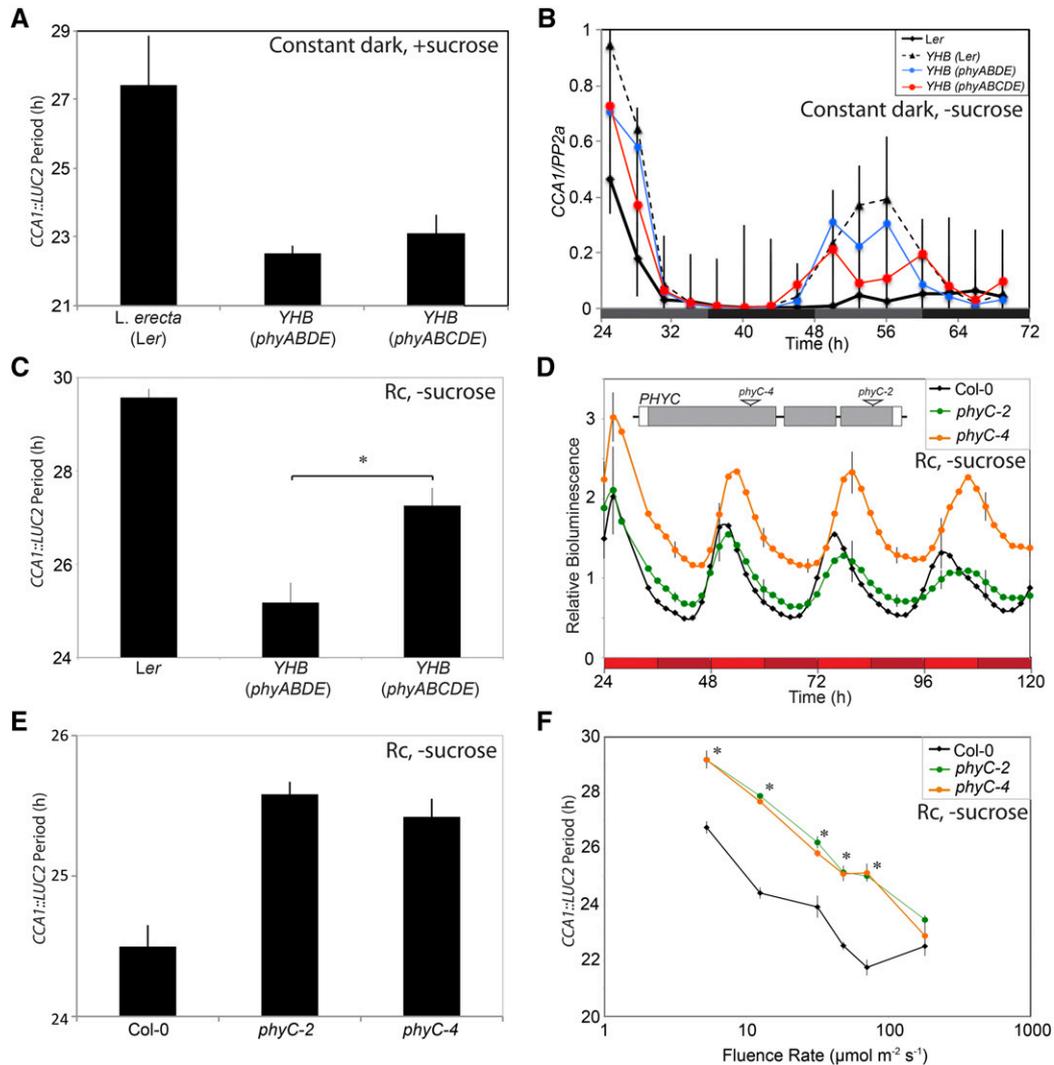
We have assessed circadian clock function in *YHB*-expressing seedlings, allowing us to evaluate the effects of a single active phy species on the circadian system independently from light effects on photosynthesis. The YHB mimic of light-activated phyB was sufficient to



**Figure 6.** YHB in the absence of other phys influences clock gene expression. A to D, Abundance of circadian transcripts under constant darkness in *YHB* seedlings in the presence or absence of native phys using qRT-PCR. Levels of *CCA1* (A), *PRR9* (B), *GI* (C), and *ELF4* (D) mRNA were assessed. Plants were entrained for 10 d in 12:12 L/D cycles on Suc-free MS media with  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light before transfer to constant darkness. mRNA levels for each gene were normalized to *PP2a*; SEM is shown. E, Circadian periodicity of *phyABCDE*, *YHB(phyABCDE)*, and *YHB-G767R(phyABCDE)* seedlings expressing a *CCA1::LUC2* reporter when grown on MS + Suc plates. Plants were entrained for 6 d under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles before being transferred to constant darkness at ZT12. Bioluminescence from groups of five seedlings was pooled for each data point,  $n > 8$ . SEM is shown. \*, Significant difference ( $P = 0.0016$ , Student's *t* test).

sustain high-amplitude, rhythmic accumulation of *CCA1*, *PRR9*, *TOC1*, and *GI* transcripts in constant darkness in the absence of exogenous sugar in both *Col* and *Ler* accessions (Figs. 2, E and G, 3, and 6). This may be due to the increased expression of *ELF4* in *YHB* plants, which shows a robust peak of expression on the first subjective day of free run in this genotype but not in the wild type (Figs. 3D and 6D). This difference precedes the first observed difference in cyclic amplitude in *CCA1* and *PRR9* transcripts in *YHB* and control plants, which is not seen until the morning of the second subjective day

in free run (Figs. 2G, 3A, and 6, A and B). *ELF4* forms part of the Evening Complex (Nusinow et al., 2011), which directly represses expression of clock genes such as *PRR7*, *PRR9*, *GI*, and *ARRHYTHMO* (*LUX*; Herrero et al., 2012; Mizuno et al., 2014; Box et al., 2015). Intriguingly, *ELF4* also is necessary for red light-mediated induction of *CCA1* and *LHY* (Kikis et al., 2005). We therefore suggest that sustained high-amplitude expression of Evening Complex components contributes to the maintenance of transcriptional rhythms in *YHB* plants in the dark.



**Figure 7.** PhyC modulates light input into the circadian system. A, Circadian periodicity of *Ler*, *YHB(phyABDE)*, and *YHB(phyABCDE)* seedlings transformed with *CCA1::LUC2* after transfer to constant darkness. Plants were grown under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles for 6 d with supplemental Suc before being transferred to constant darkness at ZT12. Bioluminescence from groups of five seedlings was pooled for each data point,  $n > 9$ . B, Abundance of *CCA1* transcripts under constant darkness in *YHB(ABDE)* and *YHB(ABCDE)* seedlings using qRT-PCR. Plants were entrained to 12:12 L/D cycles on Suc-free MS medium under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light for 10 d before transfer to constant darkness at ZT12. mRNA levels for each gene were normalized to *PP2a*; SEM is shown. C, Circadian periodicity of *Ler*, *YHB(phyABDE)*, and *YHB(phyABCDE)* seedlings transferred to dim red light ( $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seedlings were grown on  $0.5\times$  Suc-free MS medium and entrained for 6 d in 12:12 L/D cycles under  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  white light before being transferred to Rc. Bioluminescence from groups of five seedlings was pooled for each data point,  $n > 7$ . D, Period estimates of *Col-0*, *phyC-2*, and *phyC-4* seedlings under Rc. Plants were entrained in 12:12 L/D cycles for 6 d before transfer to  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  Rc. The insert shows a schematic illustration of the *PHYC* locus indicating transfer DNA (T-DNA) insertion locations for *phyC-2* and *phyC-4*. 5' and 3' untranslated regions are shown in white boxes, and exons are shown in gray. T-DNA insertion points are indicated with white triangles. E, Period estimates of seedlings transformed with a *CCA1::LUC2* reporter. Wild-type (*Col-0*), *phyC-2*, and *phyC-4* seedlings were entrained as described in (D) before being transferred to  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  Rc. F, Fluence rate response curve to evaluate the effect of phyC on the free-running period of the circadian system. Wild-type (*Col-0*, black line), *phyC-2* (green), and *phyC-4* (orange) were entrained as described in D before being transferred to Rc at the indicated fluence rate. SEM is shown. \*, Significant difference from the wild type (Bonferroni-adjusted Student's *t* test).

Although luciferase and transcript oscillations were observed in *YHB* lines in constant darkness, the activity of *YHB* was not sufficient to prevent lengthening of the circadian period under these conditions when compared with even dim red light. We observed periods of

26 h in *YHB CCA1::LUC2* reporter lines (Fig. 2D), and the later phase of peak *CCA1* transcript accumulation also suggests a longer-than-24-h period in *YHB* seedlings in both *Col* and *Ler* accessions (Figs. 2G and 6A). *CCA1::LUC2* activity rhythms in dark-adapting *YHB*

plants grown with Suc are shorter ( $\tau = 24.8 \pm 0.3$  h; Fig. 2B) than in YHB plants grown in the absence of Suc ( $26.0 \pm 0.23$  h; Fig. 2D), although the mechanism underlying this difference in period remains unclear. Similarly, *PRR9* transcript oscillation was also sustained by YHB in darkness and displayed an advanced phase of peak accumulation compared with the wild-type control (Figs. 3A and 6B). Whether these *PRR9* phase advances are due to underlying differences in periodicity rather than phase will be the subject of future investigations. Notably, YHB enhances rhythms in transcript abundance even in the absence of other phys (Fig. 6). Taken together, our data establish that YHB sustains robust clock function in the absence of photosynthesis and in the absence of light activation of other photoreceptors, including phys.

### Dissecting the Role of Phys as Light Inputs to the Circadian System

The circadian period lengths of many diurnal species, including plants, are shortened in response to higher fluence rates of constant light, a phenomenon known as Aschoff's rule (Aschoff, 1960; Somers et al., 1998; Devlin and Kay, 2000). This pattern is apparent in the red light fluence rate response curves presented here (Figs. 4A and 7F). Rhythmic amplitude of luciferase activity tended to increase with fluence rate (Fig. 4C), similar to the enhancement caused by added Suc (Fig. 2, A and C; Dalchau et al., 2011). Even low fluence rates of Rc caused maximal period shortening in YHB plants, suggesting full activation of phy signaling pathways to the clock even under dim light conditions (Fig. 4A).

Our analysis clearly indicates that YHB activity sustains phy-signaling input into the circadian system in darkness regardless of the presence of exogenous Suc. However, in continuous light, it is also clear that the clock receives additional red light-derived signaling cues from other phys, from the effects of light-driven chlorophyll synthesis, and/or from metabolic changes induced by photosynthesis itself (Hu et al., 2013). *PhyA*, *phyB*, and *phyD* have each been shown to contribute to light perception by the circadian system (Somers et al., 1998; Devlin and Kay, 2000). Recent studies reveal that *phyABDE* and *phyABCDE* mutants have indistinguishable circadian phenotypes (Hu et al., 2013), consistent with the evidence that phyC protein is unstable in the absence of other phys (Clack et al., 2009). The current study defines a role for phyC within the circadian system by demonstrating both a circadian phenotype in *phyC* mutants and modulation of YHB activity by phyC (Fig. 7). The long-period phenotype of *phyC* mutants across a range of fluence rates (Fig. 7F) suggests that phyC also contributes to red light signaling into the clock, consistent with previous reports describing the altered morphology of *phyC* mutants (Franklin et al., 2003; Monte et al., 2003). The shorter periods of YHB (*phyABDE*) compared with YHB(*phyABCDE*) under Rc (Fig. 7C) strongly suggest that phyC activation,

presumably as the phyC( $P_{fr}$ ):YHB heterodimer, is responsible for the shorter circadian period in the YHB (*phyABDE*) line. In this regard, the slightly shorter period of dark-adapting YHB(*phyABDE*) compared with YHB(*phyABCDE*; Fig. 7A) could reflect the influence of residual phyC( $P_{fr}$ ) that had not fully reverted to phyC( $P_r$ ) at the onset of darkness. These results illustrate one consequence of the many interactions between phys that underlie the complex regulation of the circadian system by red light (Sharrock and Clack, 2004).

### Mechanistic Hypothesis for the Regulatory Role of YHB in the Circadian System

Sugars, either produced via photosynthesis or applied exogenously, can both affect the pace of the clock and act as a time-of-day cue (Dalchau et al., 2011; Haydon et al., 2013). Thus, it can be difficult to distinguish photoreceptor-mediated and metabolic effects of light on circadian clock function. The ability of the constitutively active YHB allele of *phyB* to maintain high-amplitude transcriptional rhythms in the dark in the absence of exogenous sugars (Figs. 3 and 6) demonstrates that phy signaling alone is sufficient to maintain robust clock function. Recent studies implicate light-regulated interactions of *PhyB* with a subset of nuclear clock proteins, including *CCA1*, *LHY*, *GI*, *TOC1*, *LUX*, and *ELF3* (Yeom et al., 2014). Under red light, the relative strength of some of these interactions is altered in planta, with binding to *LUX* increasing while interactions with *CCA1* and *TOC1* diminish (Yeom et al., 2014). Since all of the clock components function in the nucleus, yet require synthesis and transit through the cytosol, it is possible that interactions with phys could occur in both the nucleus and the cytosol. However, our analyses indicate that cytosolic YHB-G767R is unable to sustain circadian rhythms seen in YHB lines in constant darkness, nor does it shorten the clock period as measured by *CCA1::LUC*-dependent luminescence (Fig. 6). These activities thus appear dependent on the nuclear localization of YHB. However, YHB-G767R seems to evoke an advance in the phase of *PRR9* expression during the early stages of free run, suggesting a modest cytoplasmic role for YHB at least within this subloop of the circadian system (Fig. 6B). We speculate that this response could be due to cytosolic retention of  $P_{fr}$ -interacting factors such as *TOC1* that inhibit expression of *PRR9* in the nucleus (Huang et al., 2012), an intriguing possibility that we will explore in future studies.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

The pJM63-YHB<sup>8</sup> construct, genomic YHB sequence including approximately 2.3 kb native *PHYB* promoter (Su and Lagarias, 2007), was transformed into Col-0 wild type by the floral dip method. The resultant YHB<sup>8</sup>/Col line #1 was crossed with *pCCR2::LUC/Col* (Martin-Tryon et al., 2007) to obtain the YHB<sup>8</sup>/*CCR2::LUC* line. The *phyB-9/CCR2::LUC* line was generated by crossing *CCR2::LUC* plants with *phyB-9* obtained from the Arabidopsis Biological Resource Center (line CS6217). *CCA1::LUC2/Ler*, *CCA1::LUC2/YHB<sup>8</sup>/phyABDE*, *CCA1::LUC2/phyABDE*,

and *CCA1::LUC2/phyABCDE* were described previously (Hu et al., 2013). *CCA1::LUC2/YHB<sup>B</sup>/phyABCDE* was also obtained from the cross between *CCA1::LUC2/YHB<sup>B</sup>/phyABDE* and the *phyABCDE* quintuple mutant (Hu et al., 2013). *CCA1::LUC2/YHB<sup>B</sup>* was obtained from the cross between *CCA1::LUC2/Ler* and the previously reported *YHB<sup>B</sup>/Ler* line (Su and Lagarias, 2007). The pJM63-*YHB<sup>B</sup>-G767R* construct was created by site-directed mutagenesis and then transformed into *CCA1::LUC2/phyABCDE*, resulting in multiple genetically single insertion lines of *YHB-G767R/CCA1::LUC2/phyABCDE*. The *phyC-2* mutant (Monte et al., 2003) was provided by Dr. Peter Quail (Plant Gene Expression Center, Albany, CA). The *phyC-4* mutant (Salk\_007004 line) was newly isolated; it was PCR genotyped using oligonucleotides described in Supplemental Table S1. The pEarleyGate301-pCCA1::LUC2 construct (Hu et al., 2013) was transformed into Col-0, *phyC-2*, and *phyC-4* to obtain corresponding transgenic lines. Unless otherwise stated, all plants were grown under 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light with 12:12 L/D photoperiods for 6 d before transfer to the constant conditions described for each assay.

## Luciferase Imaging Assays

Plants were entrained for 6 d in 12:12 L/D cycles under white light on MS medium with or without supplemental 3% (w/v) Suc before being sprayed with 3 mM D-luciferin in 0.01% (v/v) Triton X-100. Plants were then transferred to free-running conditions under red light-emitting diodes of indicated fluence rate or held in constant darkness at ZT12 of day 6 as previously described (Jones et al., 2010). Bioluminescence from groups of 10 seedlings was pooled for each data point where seedlings were transferred to constant darkness. Imaging was completed over 5 d, and data were processed using Metamorph software (Molecular Devices). Patterns of luciferase activity were fitted to cosine waves using Fourier fast transform-nonlinear least squares (Plautz et al., 1997) to estimate circadian period length.

## qRT-PCR

RNA was isolated and qRT-PCR performed as previously described (Jones et al., 2010). Samples were run in triplicate, with starting quantity estimated from critical thresholds using the standard curve of amplification. Data for each sample were normalized to *PROTEIN PHOSPHATASE 2A (PP2a)* expression as an internal control. Primer sets used are described in Supplemental Table S1.

## Protein Extraction and Immunoblot Analysis

Dark-grown, 4-d-old seedlings were harvested for protein extraction as previously described (Su and Lagarias, 2007). After quantifying the total protein concentrations with the Pierce BCA protein assay kit (Thermo Scientific), equal amounts of proteins were separated on 4% to 20% ExpressPlus PAGE gels (GenScript) and then semidry transferred onto an Immobilon-FL PVDF membrane (EMD Millipore). PhyB and actin were immunodetected by anti-phyB B1 (gift from Dr. Peter Quail, 1:300 dilution) and anti-actin (#MA1-744, 1:1,000, Thermo Scientific) monoclonal antibodies, respectively. The IRDye 800CW goat-anti-mouse IgG (H+L) secondary antibody (LI-COR) was used to detect the primary antibodies. Immunoreactive bands were recorded by scanning the membrane with the Odyssey infrared imaging system (LI-COR).

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Analysis of YHB function in the absence of other phytochromes.

**Supplemental Figure S2.** Circadian periodicity of *phyC* mutants.

**Supplemental Table S1.** Oligos used in this study.

Received May 26, 2015; accepted July 6, 2015; published July 8, 2015.

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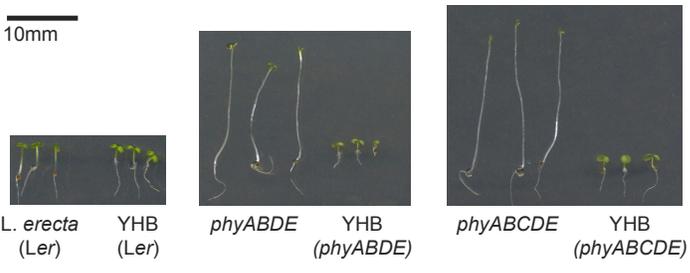
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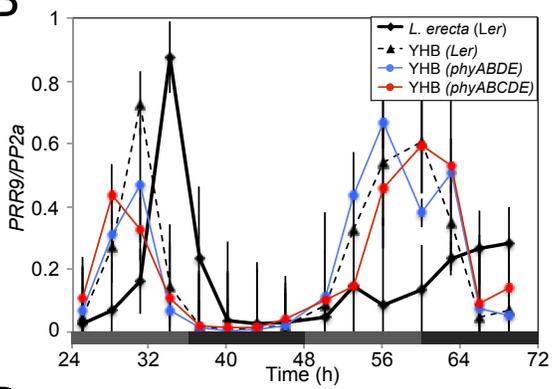
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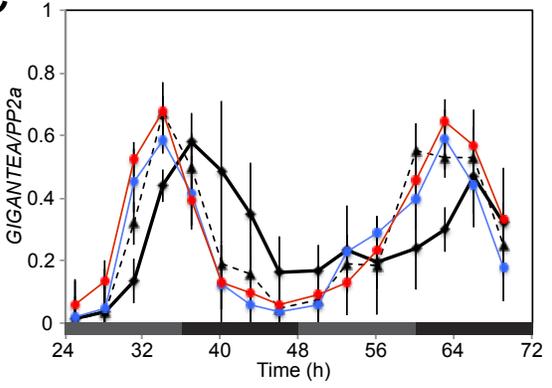
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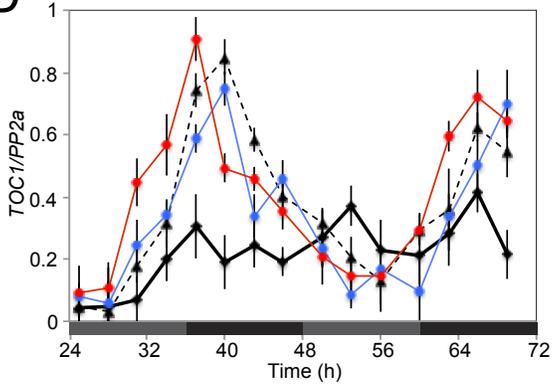
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C



D



**Supplemental Figure 1. Analysis of YHB function in the absence of other phytochromes. (A)** Morphology of seedlings transformed with *YHB*. Seedlings were grown on 0.5x MS media without sucrose for 6 days under 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light in 12:12 L/D cycles. **(B-D)** Abundance of circadian transcripts under constant darkness in *YHB* seedlings in the presence or absence of native phytochromes using qRT-PCR. Levels of *PRR9* (B), *GIGANTEA* (C) and *TOC1* (D) mRNA were assessed. Plants were entrained to 12:12 L/D cycles on sucrose-free MS media with 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light for 10 d before transfer to constant darkness at ZT12. mRNA levels for each gene were normalized to *PP2a*; SEM is shown. Gray bars indicate subjective day, black bars subjective night.



<b>Description</b>	<b>5' Oligo</b>	<b>3' Oligo</b>
<i>CCA1</i> qPCR	CAGCTCCAATATAACCGATCCAT	CAATTCGACCCTCGTCAGACA
<i>CCR2</i> qPCR	TCGTTAATGATCTTGGAATCAAT	GTATCGGTGCTTCGTTGGA
<i>PRR9</i> qPCR	GTTGAAGAGGAAAGATCGATGCTT	CTGCTCTGGTACCGAACCTTTT
<i>GI</i> qPCR	ACTAGCAGTGGTCGACGGTTTATC	GCTGGTAGACGACACTTCAATAGATT
<i>TOC1</i> qPCR	AATAGTAATCCAGCGCAATTTTCTTC	CTTCAATCTACTTTTCTTCGGTGCT
<i>ELF4</i> qPCR	GGGAGAATCTTGACCGGAAT	CAAAGCAACGTTCTTCGACA
<i>PP2a</i> qPCR	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGATTGGT
<i>phyC-4</i> T-DNA screen (WT)	TGCCAATCCTGTTACCTCAGCC	CCGCAATGCACCAATAGGTATAG
<i>phyC-4</i> T-DNA screen (insert)	TGCCAATCCTGTTACCTCAGCC	ATTTTGCCGATTTTCGGAAC

**Table S1. Oligonucleotides used in this study.**