Running Head: Phytochromes Sustain Circadian Clock Function

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Research Area: Signaling and Response

Title: **A Constitutively Active Allele of Phytochrome B Maintains Circadian Robustness in the Absence of Light**

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One Sentence Summary:

Activated phytochrome is sufficient to sustain robust circadian rhythms in the dark even in the absence of exogenous sugars, revealing the importance of light signalling pathways in clock function.

FOOTNOTES

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ABSTRACT:

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 both via photoreceptors, such as phytochromes and cryptochromes, and sugar production by photosynthesis. In the present studies, we introduce a constitutively active version of phytochrome B (phyB-Y276H, YHB) into both wild-type and phytochrome null backgrounds of *Arabidopsis thaliana* to distinguish the effects of photoreceptor signalling 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 signalling 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.

INTRODUCTION

The circadian system has evolved as an endogenous timekeeping mechanism that confines many biochemical and physiological processes to specific parts of the day and allows plants to accurately measure seasonal transitions (reviewed in Song et al., 2013; Hsu and Harmer, 2014). In order 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). While specific temperature sensors have remained elusive, light input to the circadian system occurs primarily via phytochromes, cryptochromes, and the ZTL family of photosensory F-box proteins (Somers et al., 1998; Devlin and Kay, 2000; Somers et al., 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 thaliana* (Clack et al., 1994; Franklin and Quail, 2009). Phys reversibly switch between P_r and P_{fr} 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 as well as contributing 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 (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). While 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 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 are capable of forming the homodimers necessary for signaling activity in Arabidopsis (Clack et al., 2009) or in rice (Xie et al., 2014) and instead function as heterodimers with other phytochromes.

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 feedback 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, phytochromes 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 REGULATOR 9* (*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 (RVE8) 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 quicken 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 phytochromes and other photoreceptors (Haydon et al., 2013). However, Arabidopsis seedlings lacking all five phytochromes exhibit a shorter circadian period than 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 hetero-dimers introduces complexity into the interpretation of individual phytochrome 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 phytochromes in light input to the circadian clock. In order to better understand phy-dependent and phy-independent light signaling inputs to the circadian system, we now exploit the phyB-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 phytochrome 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 (Salome 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 *CCR2::LUC* reporter (Columbia accession, Col) to measure circadian rhythms in the dark (Martin-

Tryon et al., 2007) and so we generated Columbia plants containing both the *YHB* allele and the *CCR2::LUC* reporter in addition to introducing a *CCA1::LUC2* reporter into existing *YHB* (Landsberg *erecta*; L*er*) lines (Hu et al., 2009). Introduction of the *YHB* allele confers shortened hypocotyls and expanded cotyledons in L*er* 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. 1A-B). YHB was moderately overexpressed compared to 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. 2A-D). Assessments of circadian rhythms have historically used sucrose as a media supplement in order to enhance bioluminescence in transgenic plants (Millar et al., 1992), although recent work has demonstrated that this exogenous sucrose 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 sucrose to facilitate comparison with historical and more recent datasets. We observed that rhythms of *CCR2*-driven luciferase activity in *YHB* seedlings grown with sucrose had increased amplitude throughout the experiment compared to wild-type controls ($p<0.001$, Fig. 2A), although there was no significant difference in period length between the two populations (τ=25.4±0.21 and 24.93±0.08 for wild-type and *YHB* respectively, $p=0.1$). *CCA1*-driven luciferase activity was similarly increased in *YHB* (L*er*) lines in the presence of sucrose (Fig. 2B). In the absence of sucrose, we observed that rhythmic bioluminescence in both Col wild-type and *YHB* seedlings containing a *CCR2:LUC* reporter dampened considerably and became arrhythmic following one day of constant darkness (Fig. 2C). By contrast, robust circadian rhythms were retained in *YHB CCA1::LUC2* lines grown in the absence of sucrose (τ=26.0±0.23, Fig. 2D). Since rhythmic luminescence activity was not observed from the *CCA1::LUC2* reporter control in control plants in the absence of sucrose (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 also sustains rhythmic *CCA1*-regulated luciferase activity in the absence of light and sucrose.

In order to better understand the role of exogenous sucrose 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. 2E-H). In the absence of sucrose, *CCR2* transcript accumulation in wild type mirrored the luciferase activity data, with one rhythmic peak of transcript accumulation before dampening towards arrhythmia (Fig. 2E). Rhythms were more robust in YHB plants grown without sucrose, 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 sucrose, while rhythmicity was retained in YHB-expressing lines (Fig. 2G-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 ZT12, 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 *ELF4* (Fig. 2G and Fig. 3A-D). 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 sucrose, 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 constant red light (Rc). Following an entrainment period, wild-type, *YHB* and *phyB-9* seedlings grown on sucrose-free media 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; Palagyi et al., 2010), the circadian period of wild-type plants shortened from 25.1 ± 0.33 h to 22.9 ± 0.13 h as the fluence rate increased from 12 to 184 μmol m^{-2} 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; Palagyi et al., 2010). By contrast, *YHB* seedlings were essentially unresponsive to increasing fluence rates of Rc (up to 184 μmol m^{-2} 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 wild type at the lowest fluence rate of Rc (12 μmol m^{-2} s^{-1} Rc) tested, where a ~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 μmol m^{-2} 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. 4B-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 wild type.

YHB influences clock gene expression in the absence of other phytochromes

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 to *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 hetero-dimeric 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 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. 5A-B).

To further explore the effect of YHB in the absence of other phytochromes, we assessed the accumulation of core clock gene transcripts in the *phyABCDE* mutant background using qRT-PCR. The *phyABCDE* mutant was generated in the L*er* background; hence we used a previously reported *YHB* line (*YHB*(L*er*); Su and Lagarias, 2007; Hu et al., 2009) as a control. As observed for *YHB*(L*er*) plants, *YHB*(*phyABCDE*) seedlings transferred to constant darkness displayed robustly rhythmic *CCA1* accumulation - a response that is strongly damped in L*er* wild type (Fig. 6A). *YHB*(*phyABCDE*) also sustained rhythmic expression of *PRR9*, *GI* and *ELF4* transcripts in prolonged darkness (Fig. 6B-D). All of these clock genes displayed dampened oscillations in dark-adapting L*er* wild type, similar to our results in the Col accession (Fig. 2 and Fig. 3). These data indicate that endogenous phytochromes 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 phytochrome, YHB does not require light activation to migrate from the cytoplasm to 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 PIF bHLH transcription factors and targeting them for degradation (reviewed by Bae and Choi, 2008). More recently, signaling roles for P_{fr} in the cytoplasm have been reported (Paik et al., 2012; Hughes, 2013). In order to evaluate the contribution of cytoplasmic P_{fr} signaling into the circadian system, we introduced the G767R mutation into the YHB allele (YHB-G767R). Phytochromes 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 phytochrome 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. 5A, B). However, these results contrast with the strong hyperactivity of YHB in both null and wild type backgrounds (Fig. 5A, B). Thus it is clear that the G757R mutation largely suppresses the gain-of-function activity of YHB, presumably by retaining it in the cytosol.

In order 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 sucrose (Fig. 6A-D). Whereas *YHB* expressing plants demonstrated robust rhythms in steady-state mRNA levels, dark-adapting *YHB-G767R*(*phyABCDE*) lines grown in the absence of sucrose showed rapidly damping rhythms very similar to those observed in dark-adapting wild-type L*er*.

We next examined the effects of activated phytochrome containing the G767R mutation on circadian pace. The YHB or YHB-G767 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 sucrose. While *YHB*(*phyABCDE)* plants had a shorter period of 23.68±0.08 h compared to the parental *phyABCDE* (26.57±0.48 h), the period length of *YHB-G767R*(*phyABCDE*) plants was indistinguishable from the control (27.04±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 phytochromes. 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, in order to compare the clock phenotype of these *YHB*(*phyABDE*) seedlings with that of *YHB*(*phyABCDE*) plants (Fig. 7A-C). In these two dark-adapting lines grown in the presence of sucrose, the rhythms of *CCA1::LUC2* expression were indistinguishable (Fig. 7A), with periods of 22.51±0.24 h and 23.13±0.54 h in *YHB*(*phyABDE*) and *YHB*(*phyABCDE*), respectively. Similarly, in the absence of supplementary sucrose, no significant difference in the accumulation of the clock gene transcripts *CCA1* (Fig. 7B), *PRR9*, *GI* or *TOC1* (Supplemental Fig. 1B-D) 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 sucrose (τ=25.18±0.42 and 27.26±0.38 respectively, p=0.02, Fig. 7C) suggesting that phyC can enhance YHB signalling 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 phytochromes (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. 6D-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. 2). Both *phyC-2* and *phyC*-4 seedlings exhibited longer circadian periods than wild type across a broad range of fluence rates (Fig. 7F). Such data lead 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 CONCLUSIONS

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 phytochrome species on the circadian system independently from light effects on photosynthesis. The YHB mimic of light-activated phyB was sufficient to 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 L*er* accessions (Fig. 2E, Fig. 2G, Fig. 3, and Fig. 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 wild type (Fig. 3D and Fig. 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 (Fig. 2G, Fig. 3A, Fig. 6A, 6B). ELF4 forms part of the Evening Complex (Nusinow et al., 2011) which directly represses expression of clock genes such as *PRR7*, *PRR9*, *GI*, and *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.

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 to even dim red light. We observed periods of 26 hours in *YHB CCA1::LUC2* reporter lines (Fig. 2D), and the later phase of peak *CCA1* transcript accumulation also suggests a longer-than-24-hour period in *YHB* seedlings in both Col and L*er* accessions (Fig. 2G and Fig. 6A). *CCA1::LUC2* activity rhythms in dark-adapting YHB plants grown with sucrose are shorter (τ=24.8±0.3 hrs, Fig. 2B) than in YHB plants grown in the absence of sucrose (26.0±0.23 hrs, 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 to the wild-type control (Fig. 3A and Fig. 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 phytochromes (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 phytochromes.

Dissecting the role of phytochromes 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 (Fig. 4A and Fig. 7F). Rhythmic amplitude of luciferase activity tended to increase with fluence rate (Fig. 4C), similar to the enhancement caused by added sucrose (Fig. 2A, 2C; Dalchau et al., 2011). Even low fluence rates of Rc caused maximal period shortening in YHB plants, suggesting full activation of phytochrome signalling 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 sucrose. However, in continuous light it is also clear that the clock receives additional red light-derived signaling cues, either 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 towards light perception by the circadian system (Somers et al., 1998; Devlin and Kay, 2000). Recent studies reveal *phyABDE* and *phyABCDE* mutants to have indistinguishable circadian phenotypes (Hu et al., 2013), consistent with the evidence that phyC protein is unstable in the absence of other phytochromes (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(Pfr):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 to *YHB*(*phyABCDE*) (Fig. 7A) could reflect the influence of residual phyC(Pfr) that had not fully reverted to phyC(Pr) at the onset of darkness. These results illustrate one consequence of the many interactions between phytochromes that underlie the complex regulation of the circadian system by red light (Sharrock and Clack, 2004).

Mechanistic hypothesis for the regulatory role YHB on 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 (Fig. 3 and Fig. 6) demonstrates that phytochrome signalling 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 phytochromes could occur both in 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 sub-loop of the circadian system (Fig. 6B). We speculate that this response could be due to cytosolic retention of Pfr-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*^{g} construct, genomic *YHB* sequence including ~2.3 kbp native *PHYB* promoter (Su and Lagarias, 2007), was transformed into Col-0 wild type by floral dip method. The resultant *YHB*^{g}/Col line #1, was crossed with *pCCR2::LUC*/Col (Martin-Tryon et al., 2007) to obtain the *YHB*^{g}*/CCR2::LUC* line. The *phyB-9/CCR2::LUC* line was generated by crossing *CCR2::LUC* plants with *phyB-9* obtained from the ABRC (line CS6217). *CCA1::LUC2*/L*er*, *CCA1::LUC2/YHB*^{g}*/phyABDE*, *CCA1::LUC2/phyABDE* and *CCA1::LUC2/phyABCDE* were described previously (Hu et al., 2013). *CCA1::LUC2/YHB*^{g}*/phyABCDE* was also obtained from the cross between *CCA1::LUC2/YHB*^{g}*/phyABDE* and the *phyABCDE* quintuple mutant (Hu et al., 2013). *CCA1::LUC2/YHB*^{g} was obtained from the cross between *CCA1::LUC2*/L*er* and the previously reported *YHB*^{g}*/*L*er* line (Su and Lagarias, 2007). The pJM63-*YHB*^{g}*-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 kindly provided by Dr. Peter Quail. The *phyC-4* mutant (Salk_007004 line) was newly isolated; it was PCR genotyped using oligonucleotides described in Supplemental Table 1. 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 μmol m^{-2} s^{-1} white light with 12:12 light:dark photoperiods for 6 days before transfer to the constant conditions described for each assay.

Luciferase Imaging Assays

Plants were entrained for 6 days in 12:12 light:dark cycles under white light on Murashige and Skoog (MS) media with or without supplemental 3% sucrose before being sprayed with 3 mM D-luciferin in 0.01% Triton X-100. Plants were then transferred to free-running conditions under red LEDs 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 were pooled for each data point where seedlings were transferred to constant darkness. Imaging was completed over 5 days and data was processed using Metamorph software (Molecular Devices). Patterns of luciferase activity were fitted to cosine waves using Fourier Fast Transform-Non-Linear 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 *PP2a* expression as an internal control. Primer sets used are described in Supplemental Table 1.

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 4-20% ExpressPlus™ PAGE gels (GenScript) and then semi-dry transferred onto Immobilon®-FL PVDF membrane (EMD Millipore). PhyB and actin were immunodetected by anti-phyB B1 (generous gift from Dr. Peter Quail, 1:300 dilution) and anti-actin (Thermo Scientific, #MA1-744, 1:1000) 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).

Author Contributions

MAJ, WH, JCL and SLH designed the research; MAJ, WH and SL performed research; MJ and WH contributed new tools; MAJ, WH, SL, JCL and SLH analyzed data; MJ, WH, JCL and SLH wrote the paper.

Literature cited

Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol 25: 11-28

Bae G, Choi G (2008) Decoding of Light Signals by Plant Phytochromes and Their Interacting Proteins. Ann Rev Plant Biol 59: 281-311

Bognár LK, Hall A, Adám E, Thain SC, Nagy F, Millar AJ (1999) The circadian clock controls the expression pattern of the circadian input photoreceptor, phytochrome B. PNAS 96: 14652-14657

Box MS, Huang BE, Domijan M, Jaeger KE, Khattak AK, Yoo SJ, Sedivy EL, Jones DM, Hearn TJ, Webb AAR, Grant A, Locke JCW, Wigge PA (2015) ELF3 controls thermoresponsive growth in Arabidopsis. Current Biology 25: 194-199

Casal JJ (2013) Photoreceptor signaling networks in plant responses to shade. Annual Rev Plant Biol 64: 403-427

Casal JJ, Candia AN, Sellaro R (2014) Light perception and signalling by phytochrome A. J Exp Bot 65: 2835-2845

Chen A, Li C, Hu W, Lau MY, Lin H, Rockwell NC, Martin SS, Jernstedt JA, Lagarias JC, Dubcovsky J (2014) Phytochrome C plays a major role in the acceleration of wheat flowering under long-day photoperiod. PNAS 111: 10037-10044

Clack T, Mathews S, Sharrock RA (1994) The phytochrome apoprotein family in Arabidopsis is encoded by five genes: the sequences and expression of PHYD and PHYE. Plant Mol Biol 25: 413-427

Clack T, Shokry A, Moffet M, Liu P, Faul M, Sharrock RA (2009) Obligate heterodimerization of Arabidopsis phytochromes C and E and interaction with the PIF3 basic helix-loop-helix transcription factor. Plant Cell 21: 786-799

Dalchau N, Baek SJ, Briggs HM, Robertson FC, Dodd AN, Gardner MJ, Stancombe MA, Haydon MJ, Stan G-B, Gonçalves JM, Webb AAR (2011) The circadian oscillator gene GIGANTEA mediates a long-term response of the Arabidopsis thaliana circadian clock to sucrose. PNAS 108: 5104-5109

Devlin P, Kay S (2000) Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell 12: 2499-2510

Fankhauser C, Staiger D (2002) Photoreceptors in Arabidopsis thaliana: light perception, signal transduction and entrainment of the endogenous clock. Planta 216: 1-16

Farinas B, Más P (2011) Functional implication of the MYB transcription factor RVE8/LCL5 in the circadian control of histone acetylation. Plant J 66: 318-329

Farré EM, Harmer SL, Harmon FG, Yanovsky MJ, Kay SA (2005) Overlapping and distinct roles of PRR7 and PRR9 in the Arabidopsis circadian clock. Current Biology 15: 47-54
Fogelmark K, Troein C (2014) Rethinking Transcriptional Activation in the Arabidopsis Circadian Clock. PLoS Computational Biology 10: e1003705
Franklin K, Davis S, Stoddart W, Vierstra R, Whitelam G (2003) Mutant analyses define multiple roles for phytochrome C in Arabidopsis photomorphogenesis. Plant Cell 15: 1981-1989
Franklin KA, Quail PH (2009) Phytochrome functions in Arabidopsis development. J Exp Bot 61: 11-24
Gendron JM, Pruneda-Paz JL, Doherty CJ, Gross AM, Kang SE, Kay SA (2012) Arabidopsis circadian clock protein, TOC1, is a DNA-binding transcription factor. PNAS 109: 3167-3172
Harmer SL (2009) The circadian system in higher plants. Ann Rev Plant Biol 60: 357-377
Haydon MJ, Mielczarek O, Robertson FC, Hubbard KE, Webb AAR (2013) Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. Nature 502: 689-692
Herrero E, Kolmos E, Bujdoso N, Yuan Y, Wang M, Berns MC, Uhlworm H, Coupland G, Saini R, Jaskolski M, Webb A, Gonçalves J, Davis SJ (2012) EARLY FLOWERING4 recruitment of EARLY FLOWERING3 in the nucleus sustains the Arabidopsis circadian clock. Plant Cell 24: 428-443
Hsu PY, Devisetty UK, Harmer SL (2013) Accurate timekeeping is controlled by a cycling activator in Arabidopsis. eLife 2: e00473
Hsu PY, Harmer SL (2014) Wheels within wheels: the plant circadian system. Trends in Plant Science 19: 240-249
Hu W, Franklin KA, Sharrock RA, Jones MA, Harmer SL, Lagarias JC (2013) Unanticipated regulatory roles for Arabidopsis phytochromes revealed by null mutant analysis. PNAS 110: 1542-1547
Hu W, Su Y-S, Lagarias JC (2009) A light-independent allele of phytochrome B faithfully recapitulates photomorphogenic transcriptional networks. Mol Plant 2: 166-182
Huang W, Perez-Garcia P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P (2012) Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. Science 336: 75-79
Hughes J (2013) Phytochrome cytoplasmic signaling. Ann Rev Plant Biol 64: 377-402
Ito S, Matsushika A, Yamada H, Sato S, Kato T, Tabata S, Yamashino T, Mizuno T (2003) Characterization of the APRR9 pseudo-response regulator belonging to the APRR1/TOC1 quintet in Arabidopsis thaliana. Plant Cell Physiol 44: 1237-1245
Jones MA (2009) Entrainment of the Arabidopsis circadian clock. J Plant Biol 52: 202-209
Jones MA, Covington MF, DiTacchio L, Vollmers C, Panda S, Harmer SL (2010) Jumonji domain protein JMJD5 functions in both the plant and human circadian systems. PNAS 107: 21623-21628
Jones MA, Williams BA, McNicol J, Simpson CG, Brown JWS, Harmer SL (2012) Mutation of Arabidopsis spliceosomal timekeeper locus1 causes circadian clock defects. Plant Cell 24: 4066-4082
Kaczorowski KA, Quail PH (2003) Arabidopsis PSEUDO-RESPONSE REGULATOR7 Is a Signaling Intermediate in Phytochrome-Regulated Seedling Deetiolation and Phasing of the Circadian Clock. Plant Cell 15: 2654-2665
Kikis EA, Khanna R, Quail PH (2005) ELF4 is a phytochrome-regulated component of a negative-feedback loop involving the central oscillator components CCA1 and LHY. Plant J 44: 300-313
Locke J, Southern M, Kozma-Bognar L, Hibberd V, Brown P, Turner M, Millar A (2005) Extension of a genetic network model by iterative experimentation and mathematical analysis. Mol Syst Biol 1: 2005 0013
Martin-Tryon E, Kreps J, Harmer S (2007) GIGANTEA acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation. Plant Physiol 143: 473-486
Matsushika A, Makino S, Kojima M, Mizuno T (2000) Circadian Waves of Expression of the APRR1/TOC1 Family of Pseudo-Response Regulators in Arabidopsis thaliana: Insight into the Plant Circadian Clock. Plant Cell Physiol. 41: 1002-1012
Matsushita T, Mochizuki N, Nagatani A (2003) Dimers of the N-terminal domain of phytochrome B are functional in the nucleus. Nature 424: 571-573
Millar AJ, Carre IA, Strayer CA, Chua NH, Kay SA (1995) Circadian clock mutants in Arabidopsis identified by luciferase imaging. Science 267: 1161-1163
Millar AJ, Short SR, Chua NH, Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell 4: 1075-1087

Mizuno T, Nomoto Y, Oka H, Kitayama M, Takeuchi A, Tsubouchi M, Yamashino T (2014) Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in Arabidopsis thaliana. Plant & Cell Phys 55: 958-976

Monte E, Alonso JM, Ecker JR, Zhang Y, Li X, Young J, Austin-Phillips S, Quail PH (2003) Isolation and characterization of phyC mutants in Arabidopsis reveals complex crosstalk between phytochrome signaling pathways. Plant Cell 15: 1962-1980

Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua N-H, Sakakibara H (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. Plant Cell 22: 594-605

Ni M, Tepperman JM, Quail PH (1999) Binding of phytochrome B to Its nuclear signalling partner PIF3 is reversibly Induced by light. Nature 400: 781-783

Nusinow DA, Helfer A, Hamilton EE, King JJ, Imaizumi T, Schultz TF, Farré EM, Kay SA (2011) The ELF4-ELF3-LUX complex links the circadian clock to diurnal control of hypocotyl growth. Nature 475: 398-402

Paik I, Yang S, Choi G (2012) Phytochrome regulates translation of mRNA in the cytosol. PNAS 109: 1335-1340

Palagyi A, Terecskei K, Adam E, Kevei E, Kircher S, Mérai Z, Schäfer E, Nagy F, Kozma-Bognár L (2010) Functional analysis of amino-terminal domains of the photoreceptor phytochrome B. Plant Physiol 153: 1834-1845

Pfeiffer A, Nagel M-K, Popp C, Wüst F, Bindics J, Viczián A, Hiltbrunner A, Nagy F, Kunkel T, Schäfer E (2012) Interaction with plant transcription factors can mediate nuclear import of phytochrome B. PNAS 109: 5892-5897

Plautz JD, Straume M, Stanewsky R, Jamison CF, Brandes C, Dowse HB, Hall JC, Kay SA (1997) Quantitative analysis of Drosophila period gene transcription in living animals. J Biol Rhythms 12: 204-217

Pokhilko A, Fernández AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ (2012) The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. Mol Syst Biol 8: 574

Pudasaini A, Zoltowski BD (2013) Zeitlupe senses blue-light fluence to mediate circadian timing in Arabidopsis thaliana. Biochemistry 52: 7150-7158

Rockwell NC, Su Y-S, Lagarias JC (2006) Phytochrome structure and signaling mechanisms. Ann Rev Plant Biol 57: 837-858

Salome P, Xie Q, McClung C (2008) Circadian timekeeping during early Arabidopsis development. Plant Physiol 147: 1110-1125

Schaffer R, Ramsay N, Samach A, Corden S, Putterill J (1998) The late elongated hypocotyl mutation of Arabidopsis disrupts circadian rhythms and the photoperiodic control of flowering. Cell 93: 1219-1229

Sharrock RA, Clack T (2004) Heterodimerization of type II phytochromes in Arabidopsis. PNAS 101: 11500-11505

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

Somers DE, Schultz TF, Milnamow M, Kay SA (2000) ZEITLUPE encodes a novel clock-associated PAS protein from Arabidopsis. Cell 101: 319-329

Song YH, Ito S, Imaizumi T (2013) Flowering time regulation: photoperiod- and temperature-sensing in leaves. Trends in plant science 18: 575-583

Strasser B, Sánchez-Lamas M, Yanovsky MJ, Casal JJ, Cerdán PD (2010) Arabidopsis thaliana life without phytochromes. PNAS 107: 4776-4781

Strayer C, Oyama T, Schultz T, Raman R, Somers D, Mas P, Panda S, Kreps J, Kay S (2000) Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog. Science 289: 768-771

Su Y-S, Lagarias JC (2007) Light-independent phytochrome signaling mediated by dominant GAF domain tyrosine mutants of Arabidopsis phytochromes in transgenic plants. Plant Cell 19: 2124-2139

Tóth R, Kevei E, Hall A, Millar AJ, Nagy F, Kozma-Bognár L (2001) Circadian Clock-Regulated Expression of Phytochrome and Cryptochrome Genes in Arabidopsis. Plant Physiol 127: 1607-1616

Wagner D, Quail PH (1995) Mutational analysis of phytochrome B identifies a small COOH-terminal-domain region critical for regulatory activity. PNAS 92: 8596-8600

Wang Z, Tobin E (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. Cell 93: 1207-1217

Wenden B, Kozma-Bognár L, Edwards KD, Hall AJW, Locke JCW, Millar AJ (2011) Light inputs shape the Arabidopsis circadian system. Plant J 66: 480-491

Whitelam GC, Devlin PF (1997) Roles of different phytochromes in Arabidopsis photomorphogenesis. Plant Cell & Environ 20: 752-758

Woods DP, Ream TS, Minevich G, Hobert O, Amasino RM (2014) PHYTOCHROME C is an essential light receptor for photoperiodic flowering in the temperate grass, Brachypodium distachyon. Genetics 198: 397-408

Xie X, Kagawa T, Takano M (2014) The phytochrome b/phytochrome C heterodimer is necessary for phytochrome C-mediated responses in rice seedlings. PLoS ONE 9: e97264

Yeom M, Kim H, Lim J, Shin A-Y, Hong S, Kim J-I, Nam H-G (2014) How do phytochromes transmit the light quality information to the circadian clock in Arabidopsis? Mol Plant 7: 1701-1704

Figure legends

Figure 1. Characterization of an additional YHB allele. (A) Morphology of seedlings grown under 30 μmol m^{-2} s^{-1} white light in 12:12 L/D cycles. Wild type (Col-0) and Col-0 seedlings transformed with a *pPHYB::PHYB-Y276H* construct (*YHB*) were grown on MS media for 6 days. **(B)** Quantification of hypocotyl lengths of Col-0, *YHB* (Col-0), Landsberg *erecta* (L*er*) and *YHB* (L*er*) seedlings grown as described in (A). Previously described L*er* seedlings similarly transformed with YHB are presented for comparison (Su *et al.* 2007). SEM is presented, n>20. * indicates 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. L*er* wild type and various transgenic plants harboring the L*er* YHB transgenic allele derived from *YHB/phyA201phyB-5* line #5 (Su et al. 2007) are presented for comparison.

Figure 2. Effect of YHB on bioluminescence rhythms in constant darkness. (A, C) Bioluminescence of Col-0 (solid) and *YHB* (dashed) seedlings containing a *CCR2::LUC* reporter grown on MS media with (A) or without (C) exogenous sucrose; n>9. **(B, D)** Bioluminescence of L*er* (solid) and *YHB* (L*er*) (dashed) seedlings containing a *CCA1::LUC2* reporter grown on MS media with (B) or without (D) exogenous sucrose; n>10. YHB (Ler) is presented on a secondary axis in (D) for clarity. **(E, F)** Abundance of *CCR2* transcripts as measured by qRT-PCR after transfer of seedlings to constant darkness. Plants were grown with (F) or without (E) exogenous sucrose as described in (A) and (C). **(G, 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 sucrose as described in (A) and (C). Plants were entrained under 60 μmol m^{-2} s^{-1} white light in 12:12 L/D cycles for 6 days and then transferred to constant darkness (Dc) 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 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 sucrose-free media) to constant darkness. Levels of *PRR9* **(A)**, *GIGANTEA* **(B),** *TOC1* **(C)** and *ELF4* **(D)** mRNA were assessed. Plants were grown on 0.5x MS media and entrained to 12:12 L/D cycles with 60 μ mol m^{-2} 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.

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* (dashed) alleles were crossed into a Col-0 background (solid line) carrying a *CCR2::LUC* luciferase reporter. Homozygous lines were grown under 60 μmol m^{-2} s^{-1} white light 12:12 L/D cycles on 0.5x MS media for 6 days 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 μ mol m^{-2} s^{-1} Rc. Shaded red bars indicate subjective night. **(C)** Amplitude of luciferase rhythms reported in (A). SEM is shown, * indicate significant difference compared to wild-type, Bonferroni adjusted Student's t test).

Figure 5. Morphology of seedlings transformed with YHB and YHB-G767R alleles. (A) Seedlings were grown on 0.5x MS media without sucrose for 6 days under 30 μmol m^{-2} 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 HSD test).

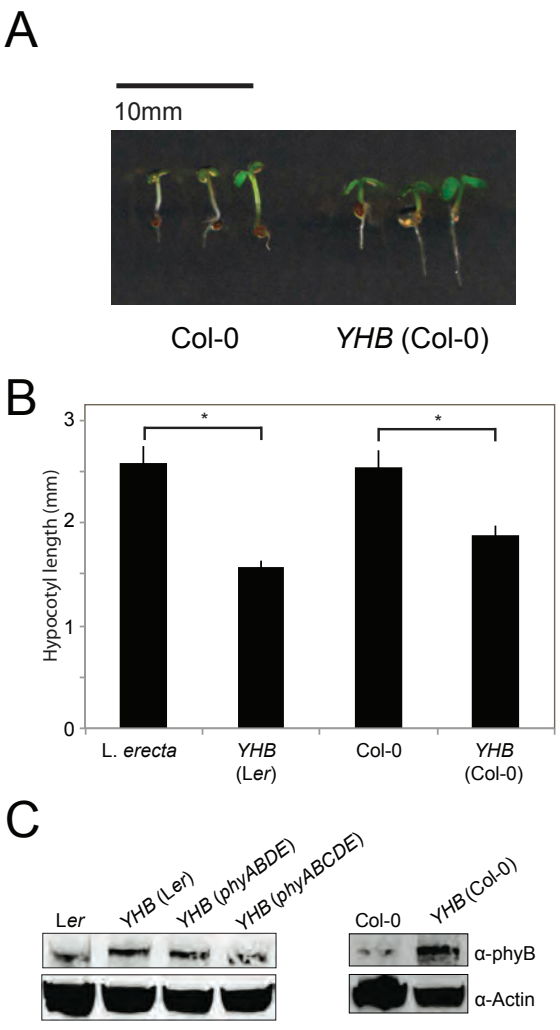
Figure 6. YHB in the absence of other phytochromes influences clock gene expression. (A-D) Abundance of circadian transcripts under constant darkness in *YHB* seedlings in the presence or absence of native phytochromes using qRT-PCR. Levels of *CCA1* (A) *PRR9* (B), *GIGANTEA* (C) and *ELF4* (D) mRNA were assessed. Plants were entrained for 10 d in 12:12 L/D cycles on sucrose-free MS media with 60 μmol m^{-2} 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 + sucrose plates. Plants were entrained for 6 days under 60 μmol m^{-2} s^{-1} white light in 12:12 L/D cycles before being transferred to constant darkness at ZT12. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, n>8. SEM is shown, * indicates significant difference ($p=0.0016$, Student's t test).

Figure 7. PhyC modulates light input into the circadian system. (A) Circadian periodicity of *L. erecta* (L*er*), *YHB*(*phyABDE*) and *YHB*(*phyABCDE*) seedlings transformed with *CCA1::LUC2* after transfer to constant darkness. Plants were grown under 60 μmol m^{-2} s^{-1} white light in 12:12 L/D cycles for 6 d with supplementary sucrose before being transferred to constant darkness at ZT12. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, 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 sucrose-free MS media under 60 μmol m^{-2} 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 μmol m^{-2} s^{-1}). Seedlings were grown on 0.5x sucrose-free MS media and entrained for 6 days in 12:12 L/D cycles under 60 μmol m^{-2} s^{-1} white light before being transferred to constant red light. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, n>7. **(D)** Period estimates of Col-0, *phyC-2* and *phyC-4* seedlings under constant red light. Plants were entrained in 12:12 L/D cycles for 6 days before transfer to 20 μmol m^{-2} s^{-1} constant red light. The insert shows a schematic cartoon of the PHYC locus indicating T-DNA insertion locations for *phyC-2* and *phyC-4*. 5' and 3' UTRs are shown in white boxes, exons in grey. T-DNA insertion points are indicated with white triangles. **(E)** Period estimates of seedlings transformed with a *CCA1::LUC2* reporter. Wild type (Col-0, solid line), *phyC-2* (green) and *phyC-4* (orange) seedlings were entrained as described in (D) before being transferred to 20 μ mol m^{-2} s^{-1}

constant red light. **(F)** Fluence rate response curve to evaluate the effect of phyC on the free running period of the circadian system. Wild type (Col-0, solid line), *phyC-2* (green) and *phyC-4* (orange) were entrained as described in (D) before being transferred to constant red light at the indicated fluence rate. SEM is shown, * indicates significant difference from wild-type (Bonferroni adjusted Student's t test).

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 μmol m^{-2} 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)**, *GI* **(C)** and *TOC1* **(D)** mRNA were assessed. Plants on sucrose-free MS media were entrained to 12:12 L/D cycles with 60 μmol m^{-2} 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.

Supplemental Figure 2. Circadian periodicity of *phyC* mutants. Independent T2 lines transformed with a *CCA1::LUC2* reporter construct were entrained on MS media for 6 days in 12:12 L/D cycles under 60 μmol m^{-2} s^{-1} before transfer to 20 μmol m^{-2} s^{-1} constant red light. Dashed lines indicate the mean period of each group of transgenics.



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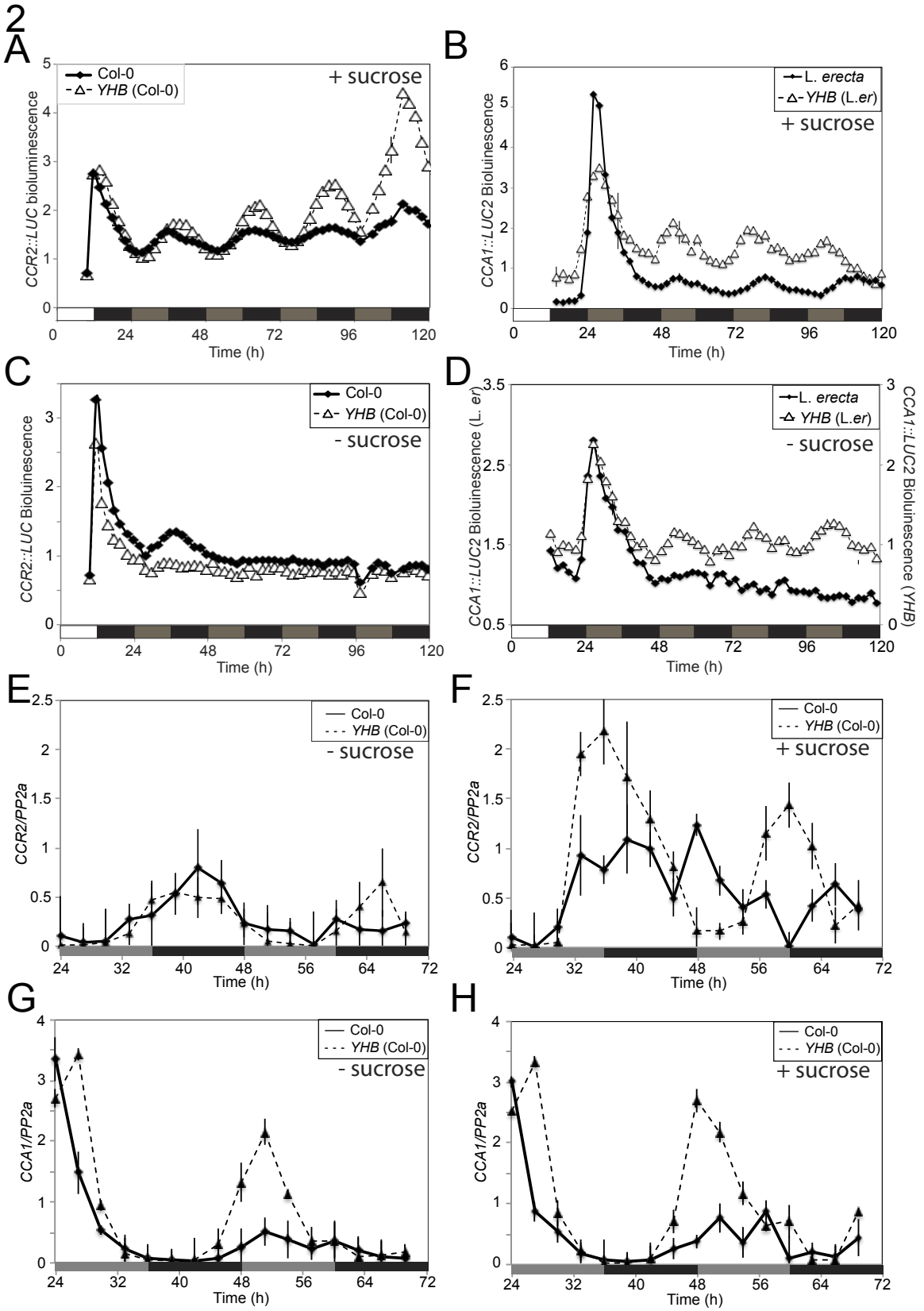

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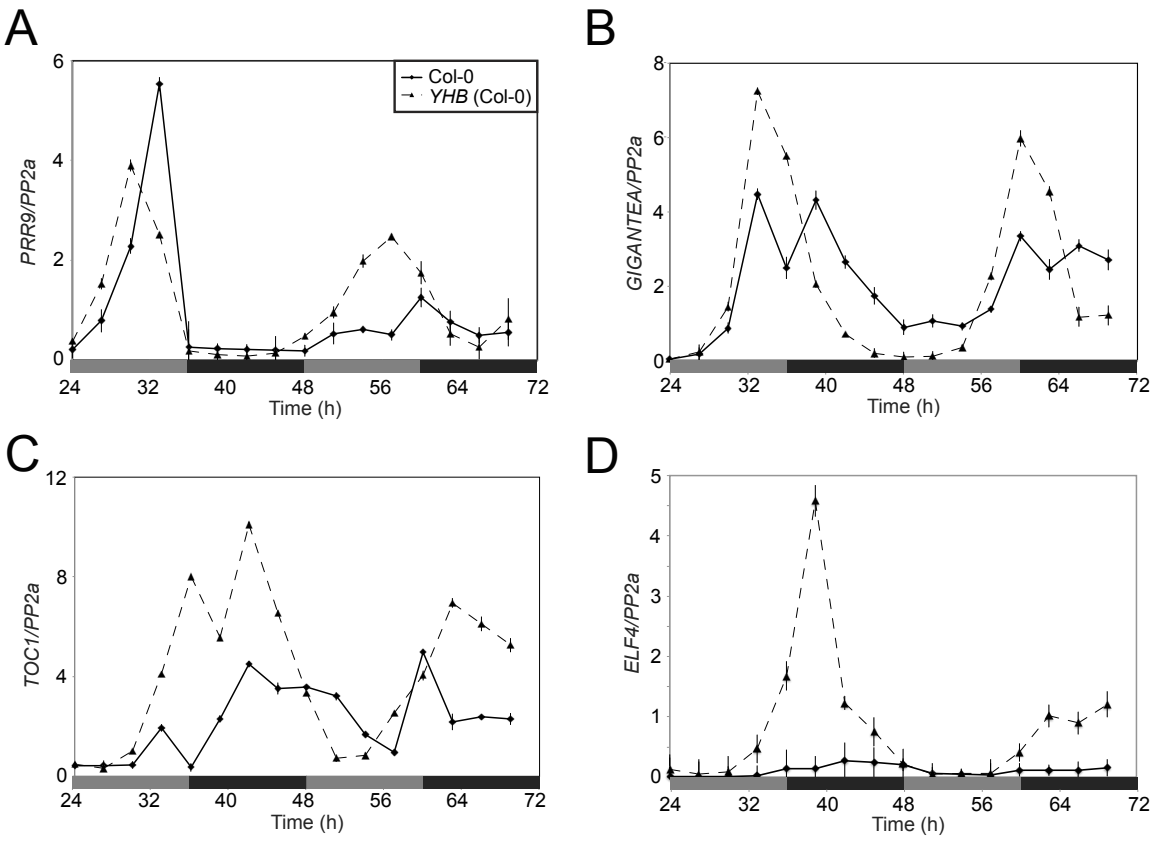

Figure 3. YHB sustains core clock transcript cycling in L/D entrained plants transferred to constant darkness. Abundance of circadian transcripts as measured by qRT-PCR after transfer of seedlings (grown on sucrose-free media) to constant darkness. Levels of *PRR9* **(A)**, *GIGANTEA* **(B),** *TOC1* **(C)** and *ELF4* **(D)** mRNA were assessed. Plants were grown on 0.5x MS media and entrained to 12:12 L/D cycles with 60 μ mol m^{-2} 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.

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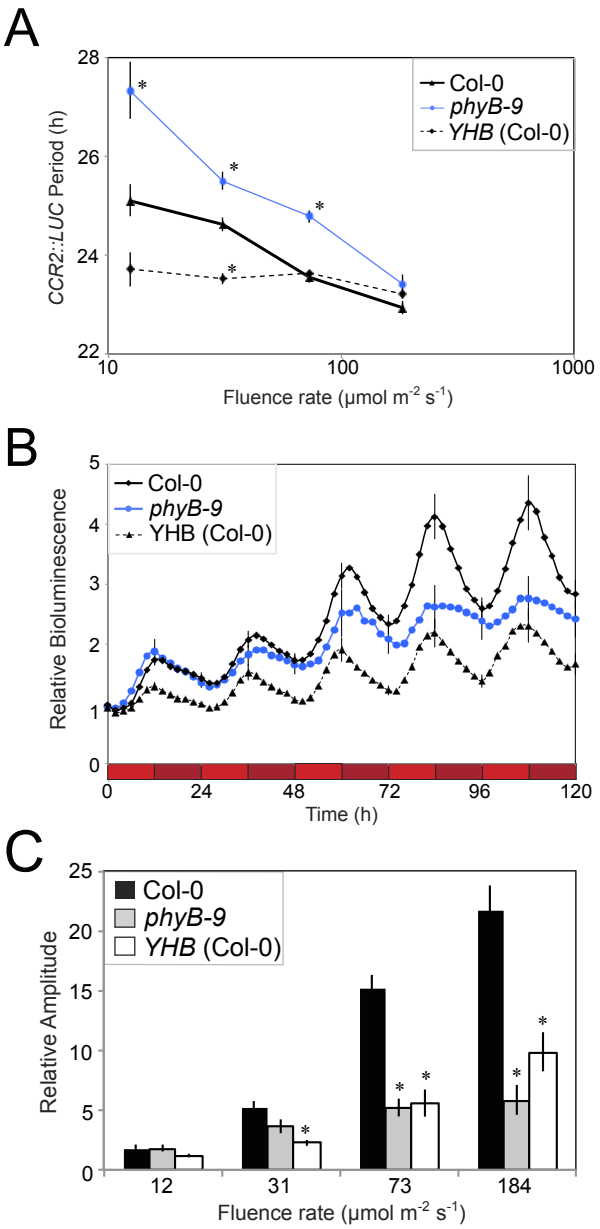

Figure 4. YHB suppresses the clock's response to increasing fluence rates of red light. (A) Fluence rate response curve to measure free-running circadian period under red light. *phyB-9* (blue) and *YHB* (dashed) alleles were crossed into a Col-0 background (solid line) carrying a *CCR2::LUC* luciferase reporter. Homozygous lines were grown under 60 µmol m^{-2} s^{-1} white light 12:12 L/D cycles on 0.5x MS media for 6 days 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 µ mol m^{-2} s^{-1} Rc. Shaded red bars indicate subjective night. **(C)** Amplitude of luciferase rhythms reported in (A). SEM is shown, * indicate significant difference compared to wild-type, Bonferroni adjusted Student's t test).

5

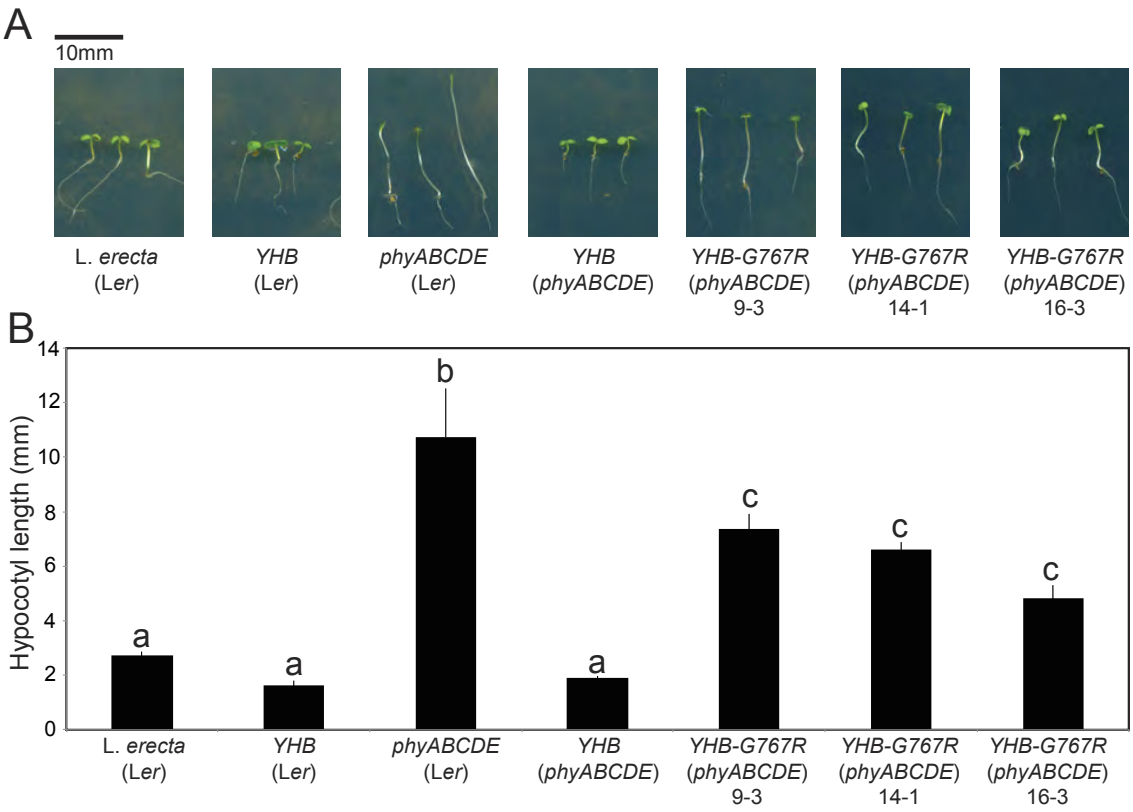

Figure 5. Morphology of seedlings transformed with YHB and YHB-G767R alleles. (A) Seedlings were grown on 0.5x MS media without sucrose for 6 days under 30 μmol m^{-2} s^{-1} white light in 12:12 L/D cycles. **(B)** Quantification of hypocotyl lengths of seedlings shown in (A). SEM is presented, n>20. Letters indicate significantly different populations ($p<0.001$, Tukey's HSD test).

6

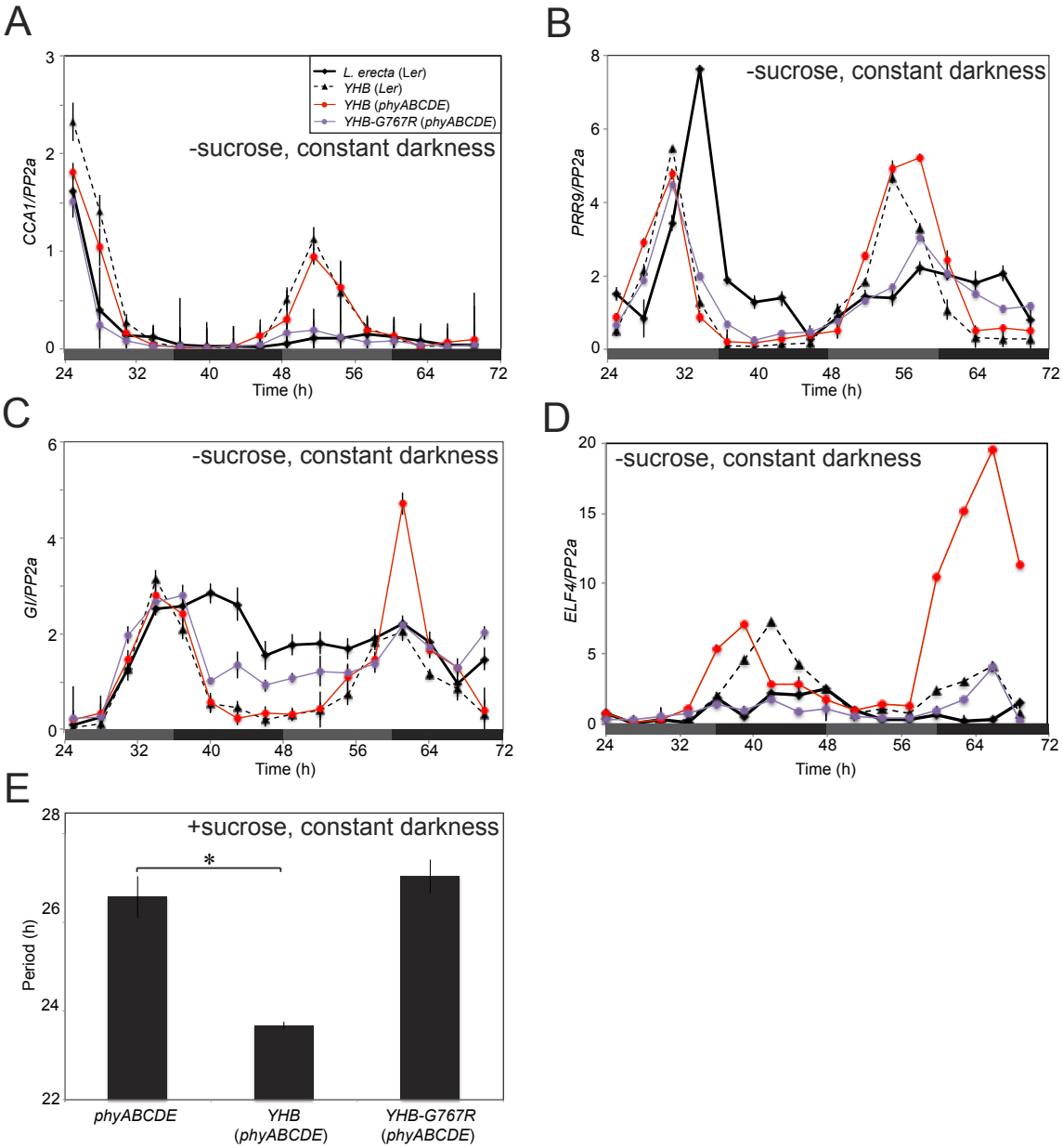

Figure 6. YHB in the absence of other phytochromes influences clock gene expression. (A-D) Abundance of circadian transcripts under constant darkness in *YHB* seedlings in the presence or absence of native phytochromes using qRT-PCR. Levels of *CCA1* (A) *PRR9* (B), *GIGANTEA* (C) and *ELF4* (D) mRNA were assessed. Plants were entrained for 10 d in 12:12 L/D cycles on sucrose-free MS media with 60 µmol m^{-2} 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 + sucrose plates. Plants were entrained for 6 days under 60 µmol m^{-2} s^{-1} white light in 12:12 L/D cycles before being transferred to constant darkness at ZT12. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, n>8. SEM is shown, * indicates significant difference (Student's t test).

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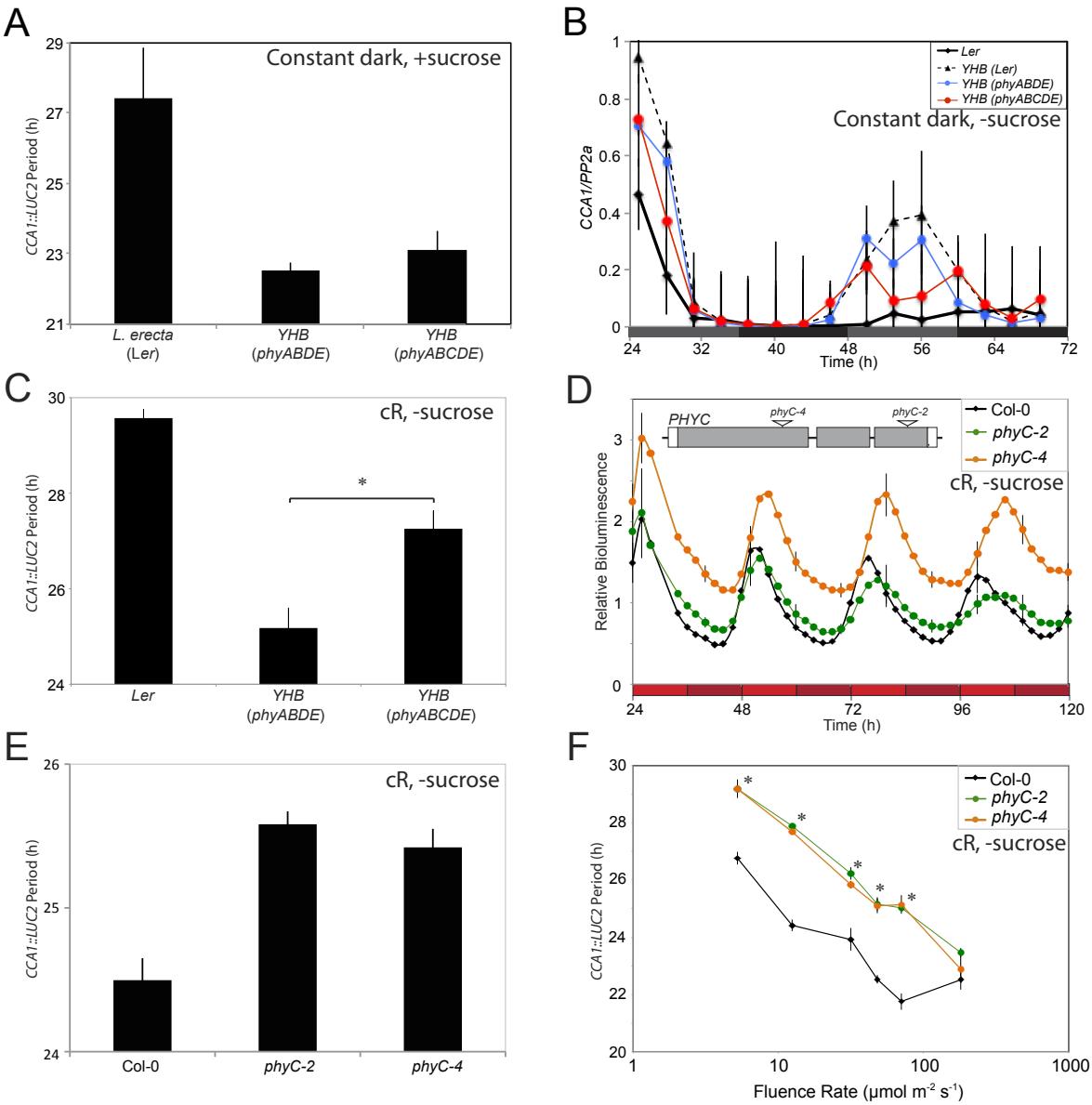
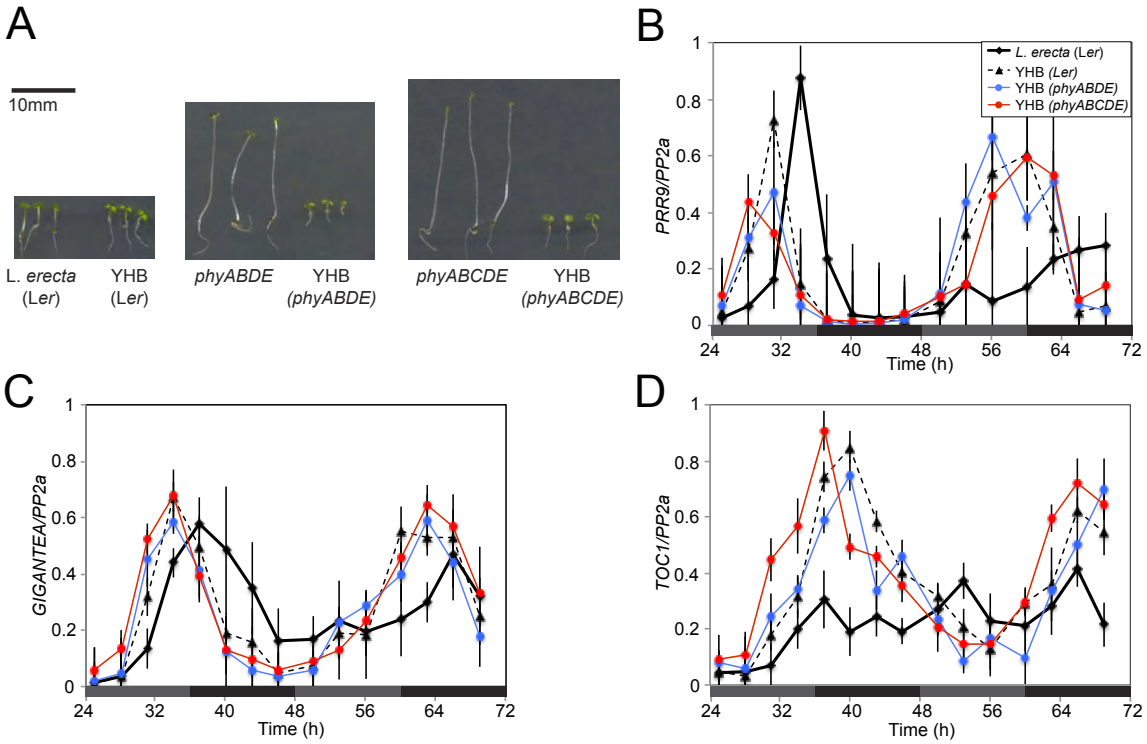


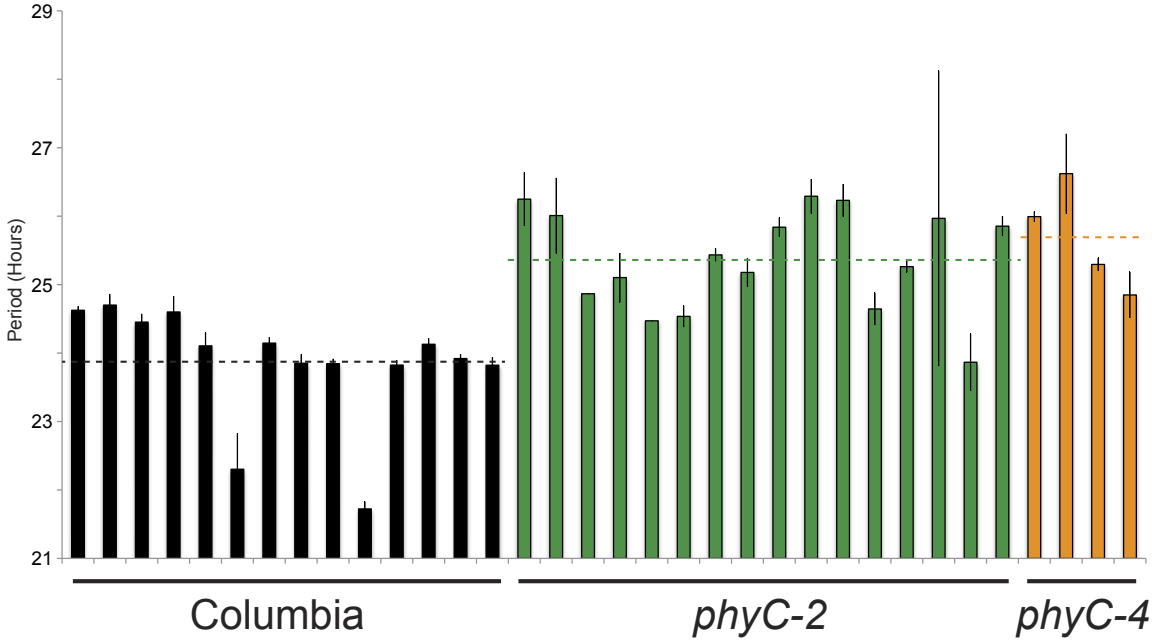
Figure 7. PhyC modulates light input into the circadian system. (A) Circadian periodicity of *L. erecta* (L*er*), *YHB*(*phyABDE*) and *YHB*(*phyABCDE*) seedlings transformed with *CCA1::LUC2* after transfer to constant darkness. Plants were grown under 60 µmol m^{-2} s^{-1} white light in 12:12 L/D cycles for 6 d with supplementary sucrose before being transferred to constant darkness at ZT12. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, 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 sucrose-free MS media under 60 µmol m^{-2} 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 µmol m^{-2} s^{-1}). Seedlings were grown on 0.5x sucrose-free MS media and entrained for 6 days in 12:12 L/D cycles under 60 µmol m^{-2} s^{-1} white light before being transferred to constant red light. Bioluminescence from groups of 5 seedlings were pooled for each datapoint, n>7. **(D)** Period estimates of Col-0, *phyC-2* and *phyC-4* seedlings under constant red light. Plants were entrained in 12:12 L/D cycles for 6 days before transfer to 20 µmol m^{-2} s^{-1} constant red light. The insert shows a schematic cartoon of the PHYC locus indicating T-DNA insertion locations for *phyC-2* and *phyC-4*. 5' and 3' UTRs are shown in white boxes, exons in grey. T-DNA insertion points are indicated with white triangles. **(E)** Period estimates of seedlings transformed with a *CCA1::LUC2* reporter. Wild type (Col-0, solid line), *phyC-2* (green) and *phyC-4* (orange) seedlings were entrained as described in (D) before being transferred to 20 µ mol m^{-2} s^{-1} constant red light. **(F)** Fluence rate response curve to evaluate the effect of phyC on the free running period of the circadian system. Wild type (Col-0, solid line), *phyC-2* (green) and *phyC-4* (orange) were entrained as described in (D) before being transferred to constant red light at the indicated fluence rate. SEM is shown, * indicates significant difference from wild-type (Bonferroni adjusted Student's t test).

S1



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 µmol m^{-2} 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 µmol m^{-2} 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.

S2



Supplemental Figure 2. Circadian periodicity of *phyC* mutants. Independent T2 lines transformed with a *CCA1::LUC2* reporter construct were entrained on sucrose-free MS media for 6 days in 12:12 L/D cycles under 60 μmol m^{-2} s^{-1} before transfer to 20 μmol m^{-2} s^{-1} constant red light. Dashed lines indicate the mean period of each group of transgenics.

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

Table S1. Oligonucleotides used in this study.