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.
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 Pr and Pfr 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; Matsuoka 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-
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; 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. 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 (Dalc hau 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 (\(\tau=25.4\pm0.21\) and 24.93\(\pm0.08\) for wild-type and YHB respectively, p=0.1). CCA1-driven luciferase activity was similarly increased in YHB (Ler) lines in the presence of 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 (\(\tau=26.0\pm0.23\), 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 *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 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 Ler 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 Ler 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 Pfr 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 Pfr in
the cytoplasm have been reported (Paik et al., 2012; Hughes, 2013). In order to evaluate the contribution of
cytoplasmic Pfr 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 Ler.
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, 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 Ler 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 Ler 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 phyts, 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-YPH 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/Col line #1 was crossed with pCCR2::LUC/Col (Martin-Tryon et al., 2007) to obtain the YHB/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/Ler, CCA1::LUC2/YHB/phyABDE, CCA1::LUC2/phyABDE and CCA1::LUC2/phyABCDE were described previously (Hu et al., 2013). CCA1::LUC2/YHB/phyABCDE was also obtained from the cross between CCA1::LUC2/YHB/phyABDE and the phyABCDE quintuple mutant (Hu et al., 2013). CCA1::LUC2/YHB was obtained from the cross between CCA1::LUC2/Ler and the previously reported YHB/Ler line (Su and Lagarias, 2007). The pJM63-YHB-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


Hsu PY, Devisetty UK, Harmer SL (2013) Accurate timekeeping is controlled by a cycling activator in Arabidopsis. eLife 2: e00473


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


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


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


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 (Ler) and YHB (Ler) seedlings grown as described in (A). Previously described Ler 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. Ler wild type and various transgenic plants harboring the Ler 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 Ler (solid) and YHB (Ler) (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
Re. 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
(Ler), 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 m² 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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  Pubmed: Author and Title
  CrossRef: Author and Title
  Google Scholar: Author Only Title Only Author and Title

  Pubmed: Author and Title
  CrossRef: Author and Title
  Google Scholar: Author Only Title Only Author and Title


Hsu PY, Devisetty UK, Harmer SL (2013) Accurate timekeeping is controlled by a cycling activator in Arabidopsis. eLife 2: e00473


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


Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua NH, Sakai K (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are...

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


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


