

SHORT COMMUNICATION

Phototropins do not alter accumulation of evening-phased circadian transcripts under blue light

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ABSTRACT

The circadian system induces rhythmic variation in a suite of biochemical and physiological processes that serve to optimise plant growth in diel cycles. To be of greatest utility, these rhythmic behaviors are coordinated with regular environmental changes such as the rising and setting of the sun. Photoreceptors, along with metabolites produced during photosynthesis, act to synchronise the internal timing mechanism with lighting cues. We have recently shown that phototropins help maintain robust rhythms of photosynthetic operating efficiency (ϕ PSII or F_q'/F_m') under blue light, although rhythmic accumulation of morning-phased circadian transcripts in the nucleus was unaffected. Here we report that evening-phased nuclear clock transcripts were also unaffected. We also observe that rhythms of nuclear clock transcript accumulation are maintained in phototropin mutant plants under a fluctuating lighting regime that induced a loss of F_q'/F_m' rhythms.

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Monitoring circadian rhythms in planta

While nuclear rhythms of gene expression are routinely measured *in planta* using luciferase reporter lines, circadian rhythms in the chloroplast can be documented by monitoring light emitted from endogenous chlorophylls following a period of illumination. Delayed Fluorescence (DF) methods monitor light emitted from chlorophyll immediately after extinguishing ambient illumination from growth lights¹ whereas comparison of chlorophyll *a* fluorescence (CaF) before and immediately after the application of a saturating light pulse allows the operating efficiency of photosystem II to be examined (ϕ PSII or F_q'/F_m' ,²⁻⁴). Variation in DF or F_q'/F_m' over time represent 2 methods that can be used to monitor circadian rhythms in the chloroplast.

Light inputs into the circadian system

To be of greatest utility the circadian system is responsive to daily and seasonal variations in photoperiod.^{5,6} Changes in ambient light and temperature signal into a biological network of interconnected feedback loops.⁵ Most work has focused upon transcription/translation feedback loops in the nucleus, but recently oscillations in protein oxidation have also been identified that continue in the absence of nuclear rhythms in certain species and tissue types.^{7,8}

Each of the identified photoreceptor families acts to either transmit information into the central circadian oscillator or modulates a circadian output.⁹ Phytochromes, cryptochromes, and UV-B RESISTANCE8 (UVR8), have been shown to accelerate nuclear clock pace in response to red, blue or UV-B

signals respectively,¹⁰⁻¹² while the role of the ZTL family in the post-translational regulation of certain circadian components in response to blue light has been well documented.^{13,14} Both distinct and converging signaling pathways initiated by these photoreceptors act on the nuclear clock although the precise mechanisms involved have yet to be elucidated in many cases.

The phototropin family of blue photoreceptors are atypical in that they have not been ascribed a role within the nuclear circadian system.^{4,15} We have recently shown that phototropins help to maintain robust rhythms of F_q'/F_m' under dim blue light, without altering rhythms in the nucleus.⁴ Here we examine the role of phototropins within the nuclear circadian system in greater detail and confirm that rhythmic transcript accumulation in the nucleus does not appear to be altered in plants lacking both phototropin1 (*phot1*) and *phot2*.

Phototropins do not alter expression levels of evening components within the circadian system

Our recent work used qRT-PCR to demonstrate that accumulation of circadian transcripts was not altered in *phot1-5 phot2-1* (*p1p2*) seedlings but our initial analysis was restricted to morning-phased transcripts.⁴ To expand our analysis, we examined the accumulation of selected evening-phased transcripts under constant blue light (Fig. 1). As for morning-phased genes, we observed no significant difference in *GIGANTEA*, *TIMING OF CAB1 EXPRESSION1* (*TOC1*), or *COLD*, *CIRCADIAN RHYTHM AND RNA BINDING2* (*CCR2*) transcript accumulation in *phot1-5*, *phot2-1* or *p1p2* double mutants compared to a wild type control (Fig. 1A-C). Initial analysis of phase and period of these rhythmic transcripts was completed using the

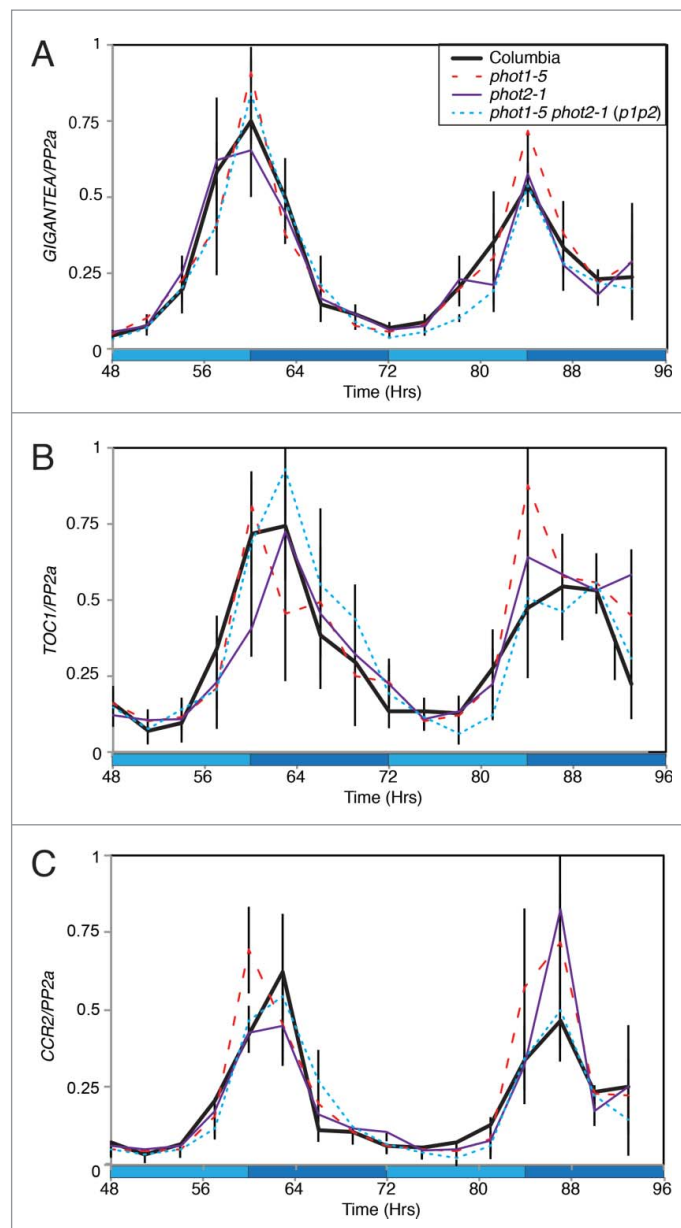


Figure 1. Accumulation of circadian clock-regulated transcripts under constant blue light. Transcript accumulation in wild type (Columbia, solid black), *phot1-5* (dashed red), *phot2-1* (purple) and *phot1-5 phot2-1* (*p1p2*, dotted blue) mutants was compared using qRT-PCR. Levels of *GIGANTEA* (A), *TOC1* (B), and *CCR2* (C) mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d on $\frac{1}{2}$ MS media before being moved to constant conditions with $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light. Data for each transcript were compared with an internal control (*PP2a*) before being normalized to the peak of wild-type accumulation. Data are the average of 3 biological replicates, error bars show standard error of the mean. Dark blue shading indicates subjective night.

JTK_CYCLE algorithm,¹⁶ although interpretation of these data are limited by the resolution and length of the qRT-PCR time course. This analysis indicated there was no difference in the phase or period in the rhythms of *GIGANTEA* transcript accumulation but minor differences were observed in relation to *TOC1* and *CCR2* transcripts. A modest 1.5 hour phase delay in *TOC1* rhythms were detected in *phot1-5* and *p1p2* seedlings that was not present in *phot2-1* while a longer 27-hour period in *TOC1* rhythms was returned for *phot2-1* and *p1p2* lines (compared to 24 hours in wild type and *phot1-5* seedlings). Peak *CCR2* transcript accumulation was also delayed by 1.5 hours, but only in *phot1-5* and *phot2-1* seedlings. Instead, *CCR2* transcripts may cycle with a longer period in *p1p2* lines (27hrs in *p1p2* compared to 24hrs in wild type and the *phot1-5*

and *phot2-1* single mutants). Although this analysis may indicate a minimal role for phototropins in the maintenance of rhythmicity of *TOC1* and *CCR2* (but not *GIGANTEA*) this proposition will need to be clarified through the use of extended qRT-PCR time courses or via luciferase reporter lines in phototropin mutant backgrounds.

The introduction of hourly dark intervals does not impair rhythmicity of the nuclear circadian clock in *p1p2* seedlings

Inclusion of an hourly dark interval into the illumination protocol during CaF imaging induced a reduction in amplitude of F_q'/F_m' rhythms under $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light

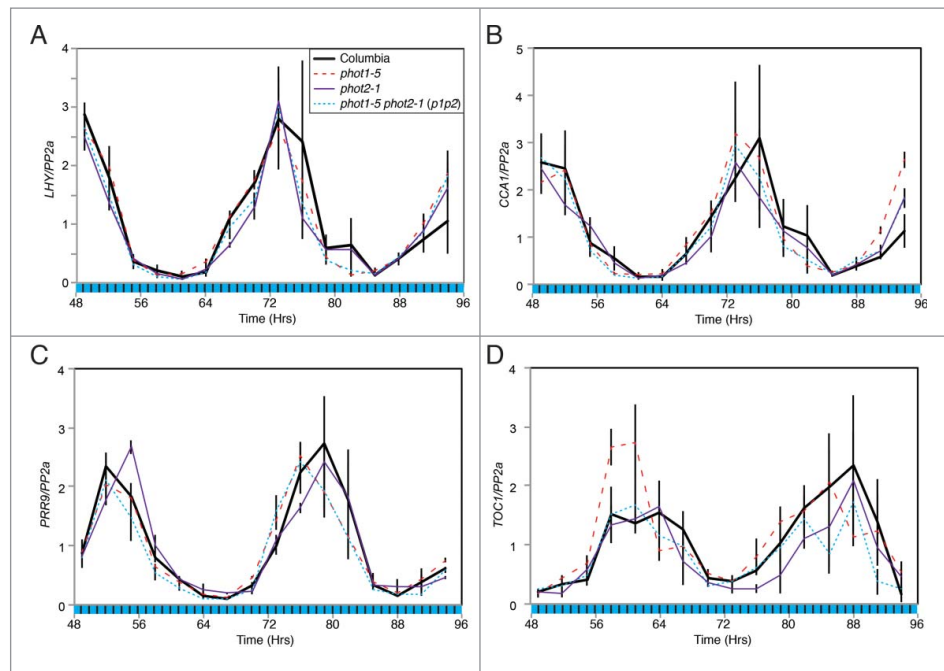


Figure 2. Accumulation of circadian clock-regulated transcripts under fluctuating blue light. Transcript accumulation in wild type (Columbia, solid black), *phot1-5* (dashed red), *phot2-1* (purple) and *phot1-5 phot2-1 (p1p2)* (dotted blue) mutants was compared using qRT-PCR. Levels of *LHY* (A), *CCA1* (B), *PRR9* (C) and *TOC1* (D) mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d on $\frac{1}{2}$ MS media before being moved to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light interspersed with 10 minute dark intervals every hour. Data for each transcript were compared with an internal control (*PP2a*) before being normalized to the peak of wild-type accumulation. Data are the average of 3 biological replicates, error bars show standard error of the mean. Black bars indicate periods of darkness during harvesting schedule.

in *p1p2* plants⁴ and so we investigated whether these conditions precipitated the loss of nuclear rhythms in these lines (Fig. 2). As under cB, we found that transcript accumulation of *LATE ELONGATED HYPOCOTYL (LHY)*, *CIRCADIAN CLOCK ASSOCIATED1 (CCA1)*, *PSEUDORESPONSE REGULATOR9 (PRR9)*, and *TOC1*, was unaltered in these conditions of fluctuating blue light (Fig. 2A-D). Such data suggest that phototropins act to maintain robust circadian oscillations of PSII operating efficiency downstream of the central nuclear oscillator and reinforce the notion that phototropins have a minimal role within the nuclear circadian system.

Defining the role of phototropins within the Arabidopsis circadian system

Phototropins are plasma-membrane localized, light-activated kinases that are re-localized to the cytosol, chloroplast outer membrane and golgi apparatus upon illumination with blue light.¹⁷⁻¹⁹ Although a nuclear localization of *phot2* has been reported as a consequence of overexpression¹⁷ examination of transgenic lines expressing *phot2* fused to GFP and a nuclear localization signal (P2G-NLS) revealed that P2G-NLS is less active than *phot2* lacking an NLS.¹⁷ Indeed, subsequent analysis revealed that a substantial proportion of P2G-NLS is retained at the plasma membrane (in addition to a nuclear population).¹⁷ It therefore remains plausible that the observed loss of activity in P2G-NLS lines is a consequence of *phot2* sequestration within the nucleus. Such data, in combination with our qRT-PCR assays suggest that phototropins act to amplify F_q'/F_m' oscillations independently of the nuclear transcription/translation circadian clock.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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