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*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 serves to optimise plant growth in diel cycles. To be of greatest utility, these rhythmic behaviours are coordinated with regular environmental changes such as the rising and setting of the sun. Photoreceptors, and 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 ( $\varphi\text{PSII}$  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.

## **TEXT**

### **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 lights<sup>1</sup> 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 ( $\varphi\text{PSII}$  or

$F_q'/F_m'$ ,<sup>2-4</sup>). Variation in DF or  $F_q'/F_m'$  over time represent two 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<sup>5,6</sup>. Changes in ambient light and temperature signal into a biological network of interconnected feedback loops<sup>5</sup>. 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<sup>7,8</sup>.

Each of the identified photoreceptor families acts to either transmit information into the central circadian oscillator or modulates a circadian output<sup>9</sup>. 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<sup>10-12</sup>, 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<sup>13,14</sup>. Both distinct and converging signalling 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<sup>4,15</sup>. 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<sup>4</sup>. 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<sup>4</sup>. To expand our analysis, we examined the accumulation of selected evening-phased transcripts under constant blue light (Figure 1). As for morning-phased genes, we observed no significant difference in *GIGANTEA*, *TIMING OF CABI 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 (Figure 1A-C). Initial analysis of phase and period of these rhythmic transcripts was completed using the JTK\_CYCLE algorithm<sup>16</sup>, 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 in *p1p2* plants<sup>4</sup> and so we investigated whether these conditions precipitated the loss of nuclear rhythms in these lines (Figure 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 (Figure 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<sup>17-19</sup>. Although a nuclear localization of phot2 has been reported as a consequence of overexpression<sup>17</sup> 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<sup>17</sup>. Indeed, subsequent analysis revealed that a substantial proportion of P2G-NLS is retained at the plasma membrane (in addition to a nuclear population)<sup>17</sup>. 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.

### **Acknowledgements**

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## Figure Legends

### 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 ½ 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 three biological replicates, error bars show standard error of the mean. Dark blue shading indicates subjective night.

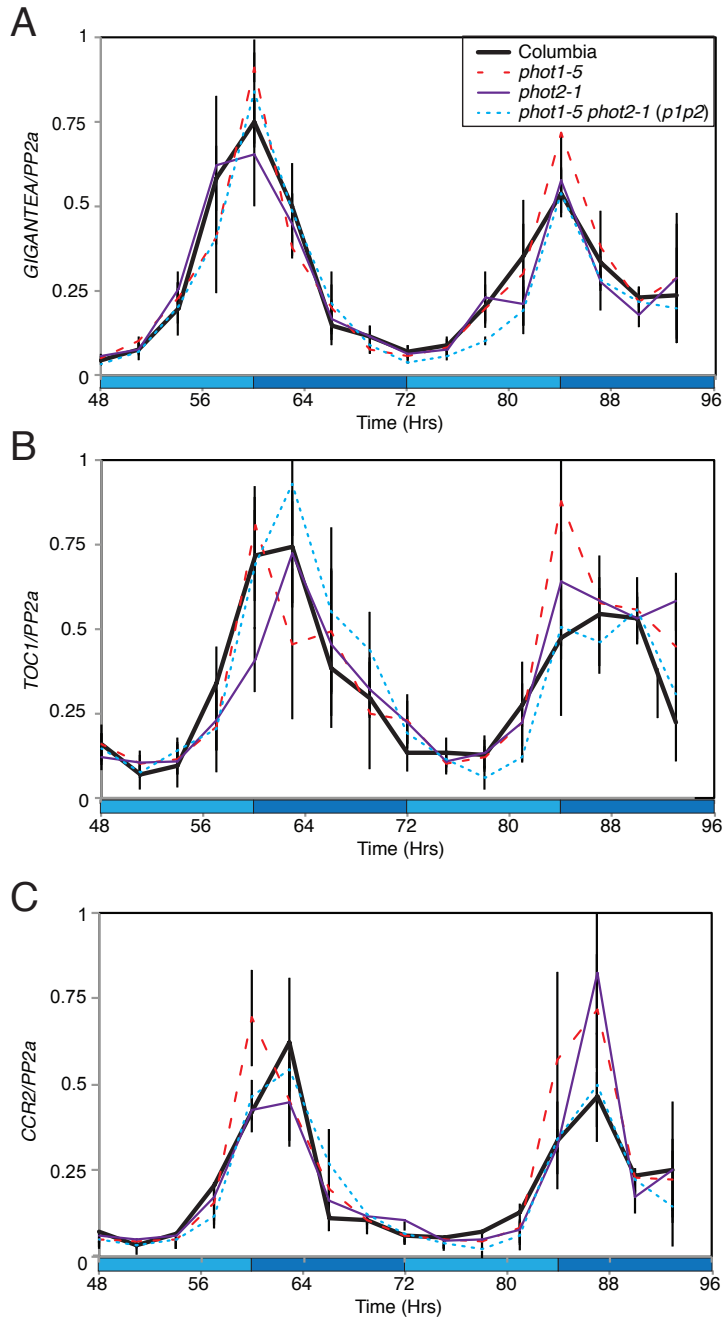
### Figure 2

#### 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

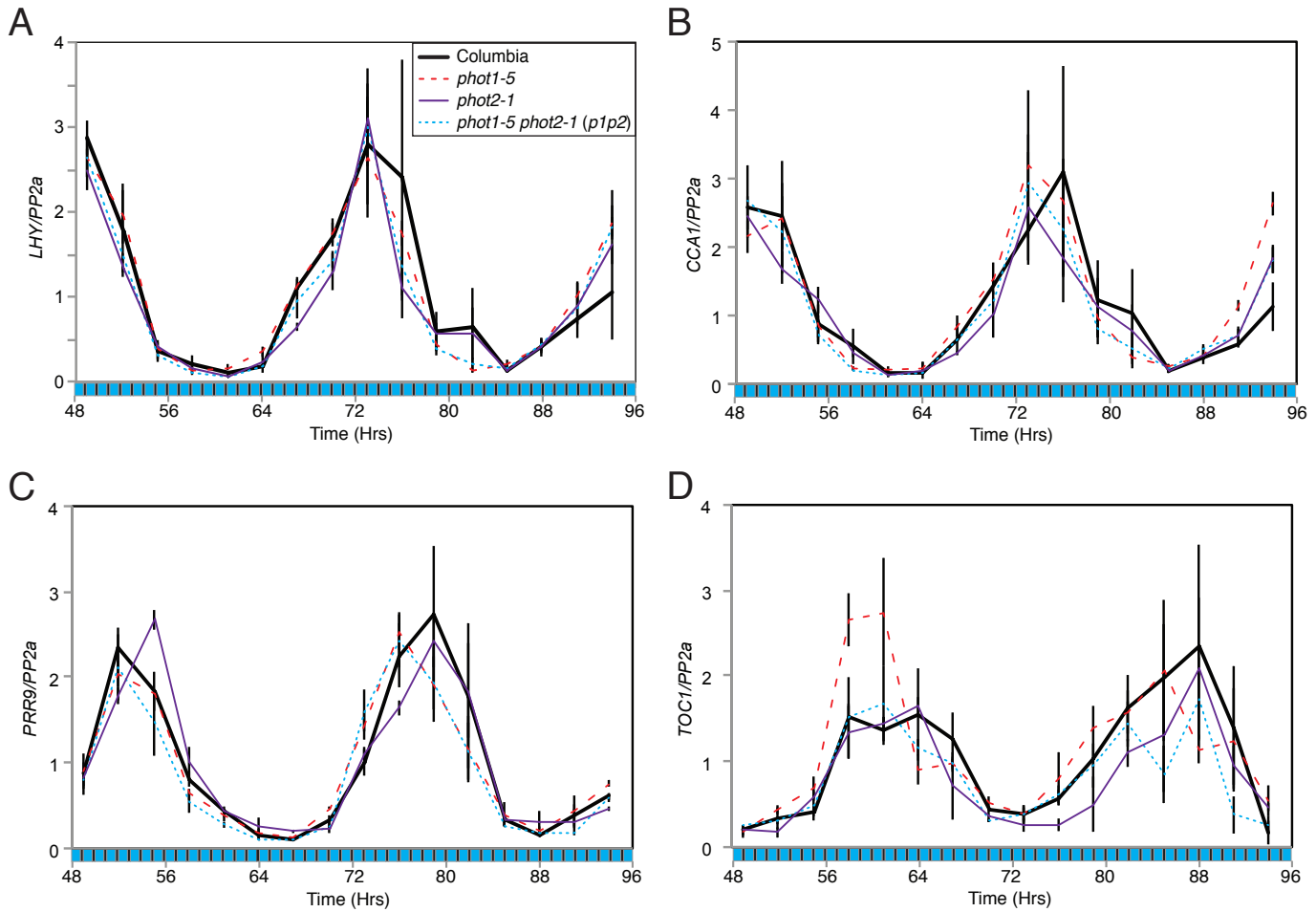
qRT-PCR. Levels of *LHY* (**A**), *CCA1* (**B**), *PRR9* (**C**) and *TOC1* (**D**) mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d on ½ MS media before being moved to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light interspersed with ten minute dark intervals every hour. Data 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 three biological replicates, error bars show standard error of the mean. Black bars indicate periods of darkness during harvesting schedule.

Figure 1



**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 ½ 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 three biological replicates, error bars show standard error of the mean. Dark blue shading indicates subjective night.

Figure 2



**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 ½ MS media before being moved to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light interspersed with dark intervals. 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 three biological replicates, error bars show standard error of the mean. Black bars indicate periods of darkness during harvesting schedule.