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Title of Article: Phototropins Maintain Robust Circadian Oscillation of PSII Operating Efficiency Under Blue Light

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Summary

The circadian system allows plants to coordinate metabolic and physiological functions with predictable environmental variables such as dusk and dawn. This endogenous oscillator is comprised of biochemical and transcriptional rhythms that are synchronized with a plant's surroundings via environmental signals, including light and temperature. We have used chlorophyll fluorescence techniques to describe circadian rhythms of PSII operating efficiency (F_q'/F_m') in the chloroplasts of *Arabidopsis thaliana*. These F_q'/F_m' oscillations appear to be influenced by transcriptional feedback loops previously described in the nucleus, and are induced by rhythmic changes in photochemical quenching over circadian time. Our work reveals that a family of blue photoreceptors, phototropins, maintain robust rhythms of F_q'/F_m' under constant blue light. As phototropins do not influence circadian gene expression in the nucleus our imaging methodology highlights differences between the modulation of circadian outputs in distinct subcellular compartments.

Significance Statement

Measurement of circadian rhythms of PSII operating efficiency in the chloroplast reveals a distinct physiological circadian output compared to previously reported delayed fluorescence methods. Phototropins are required for the maintenance of these chlorophyll fluorescence rhythms under dim blue light (while not affecting rhythms of nuclear gene expression) via a signalling cascade independent of NPH3.

Introduction

The circadian system provides a biochemical timekeeping reference that allows life to anticipate regular changes in the environment precipitated by the rotation of the Earth (Jones 2009, Hsu and Harmer 2014). In addition to inducing daily changes in plant physiology, biochemistry and gene expression the circadian oscillator is also used to correctly time longer term developmental decisions such as flowering time (Song *et al.* 2013). As such, the circadian clock has a crucial role in improving plant fitness and promoting resistance to biotic and abiotic stress (Dodd *et al.* 2005, Bhardwaj *et al.* 2011, Sanchez *et al.* 2011).

The circadian system has been succinctly described as a 'core' central oscillator that is synchronized to the environment by inputs comprising photoreceptors and temperature sensing pathways (Harmer 2009). Rhythms in the central oscillator are subsequently used to coordinate downstream processes (Hsu and Harmer 2014). While the circadian system is strongly entrained by the diurnal cycle in order to maintain synchrony with the environment, this rhythmic behaviour is retained in plants transferred to constant conditions. Impairment of light sensitivity through mutation of multiple plant photoreceptors (including phytochromes, cryptochromes, the ZEITLUPE family and UVR8) alters circadian rhythms under specific qualities of light (Somers *et al.* 1998, Devlin and Kay 2000, Kim *et al.* 2007, Baudry *et al.* 2010, Fehér *et al.* 2011). However, a role for phototropins (a family of blue light photoreceptors) within the nuclear circadian system has not yet been described (Devlin and Kay 2001).

As our knowledge of the circadian system has increased it has become apparent that there is substantial overlap between these arbitrary groupings of 'core' and 'input' clock elements. For instance, the expression of phytochrome and cryptochrome photoreceptors is regulated by the circadian system (Bognár *et al.* 1999, Harmer *et al.* 2000, Tóth *et al.* 2001), thereby blurring the definition of these proteins as input or core components. Recent models of the circadian system define the transcriptional circadian system as an interlocking series of feedback loops (Fogelmark

and Troein 2014, Hsu and Harmer 2014). *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) are expressed in the morning and repress the expression of several evening-phased clock components including *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *LUX ARRHYTHMO* (*LUX;* (Alabadi *et al.* 2001, Hazen *et al.* 2005b). TOC1 and LUX (the latter of which acts as part of the Evening Complex; Nusinow *et al.* 2011) subsequently repress *CCA1* and *LHY* expression (Alabadi*, et al.* 2001, Hazen*, et al.* 2005b), thereby forming a negative feedback loop. REVEILLE8 (RVE8) acts to promote expression of *TOC1* and *LUX* within this network (Hsu *et al.* 2013). In addition to this transcriptional network it is equally apparent that circadian oscillations occur independently of transcription, with rhythms of peroxiredoxin reduction continuing in the chloroplast in the absence of rhythmic nuclear transcription (Edgar *et al.* 2012). It is likely that the combination of circadian oscillators in different cellular compartments increase robustness and improve the benefits of the circadian system within the plant.

One of the integral metabolic processes that forms part of the extended circadian system is photosynthesis (Dodd *et al.* 2014). Plants accumulate greater biomass when their molecular clocks are in step with diurnal environmental changes (Dodd*, et al.* 2005) and CO_2 assimilation also changes over circadian time (Dodd *et al.* 2004). Timely starch degradation during the night is controlled by the clock (Graf *et al.* 2010) and levels of photosynthetically-derived sugars are able to reset the transcriptional circadian network (Haydon *et al.* 2013), illustrating how photosynthetic metabolites feedback into the transcriptional loops of the central oscillator. Circadian rhythms in the chloroplast can be monitored by measuring the residual photons emitted from the photosynthetic apparatus (referred to as Delayed Fluorescence, DF; Gould *et al.* 2009). Although the mechanisms underlying these DF rhythms remain to be determined, these findings suggest that the composition of the photosynthetic apparatus varies over circadian time (Dodd*, et al.* 2014).

Chlorophyll a fluorescence (CaF) is a non-invasive method that enables the determination of photosynthetic rates *in vivo* by monitoring re-emitted light from the leaf. Energy gathered by the photosystem II (PSII) pigment antennae may either be used for photochemistry, re-irradiated at a longer wavelength as fluorescence, or dissipated as heat (Butler 1978). Numerous studies have revealed that the parameters derived from modulated fluorescence emission, including the operating efficiency of PSII (F_q'/F_m'), are correlated with their intrinsic photosynthetic rates, particularly under non-photorespiratory conditions (Baker 2008). Here, we use CaF methods to enable the medium-throughput analysis of photosynthetic rhythms in *Arabidopsis thaliana* (Arabidopsis) under constant blue light. Application of these techniques reveals a role for phototropins in the modulation and coordination of PSII efficiency over circadian time in response to low blue light or fluctuating blue light conditions.

Results

Rhythms of photosynthetic efficiency are influenced by the nuclear circadian system

Circadian rhythms in plants are routinely measured by monitoring luciferase activity as a proxy for gene expression in transgenic plants or by delayed fluorescence, which measures residual photons emitted from the photosynthetic apparatus directly after transfer from light into darkness (Millar *et al.* 1992, Gould*, et al.* 2009, Dodd *et al.* 2014). Both of these methodologies can be used to demonstrate that Arabidopsis has a circadian period of approximately 24 hours under 20 μmol m^{-2} s^{-1} constant blue light (Figure 1a), with the phase of delayed fluorescence rhythms peaking shortly before subjective dusk (ZT11, Figure 1b). This is comparable to the circadian period estimated using luciferase imaging to visualize activity of the *CCA1* promoter (24.53±0.17 hrs with a peak at ZT3, Figure 1a-b). As the circadian system modulates many different plant behaviors we were interested how the clock altered photosynthesis. The operating efficiency of photosystem II (previously referred to as either F_q'/F_m' or ϕPSII) can be measured *in vivo* using

chlorophyll fluorescence techniques (Baker 2008) and we applied these to plants grown in constant blue light for 5 days to study how this parameter varied over circadian time (Figure 1a-h). We were able to monitor strong circadian oscillations of F_q'/F_m' with a periodicity of 24.17±0.18 hrs that was comparable to measurements by luciferase or delayed fluorescence imaging (Figure 1a). The robustness of circadian oscillations are indicated by how well the experimental data aligns with a fitted cosine wave, with a Relative Amplitude Error (RAE) of 0 indicative of a perfect fit and an RAE of 1 representing the mathematical limits of rhythm detection (Plautz *et al.* 1997). F_q'/F_m' rhythms were robust, with an average RAE of 0.18 (Figure 1a). We continued to observe F_q'/F_m' rhythms in plants where the leaves had been restrained to limit movement (Figure S1). Although periodicity in these wild type lines was comparable to previously reported measures in the chloroplast using delayed fluorescence (Figure 1a), the phasing of peak F_q'/F_m' (before subjective dawn, at ZT21) was ten hours later than the maxima observed by delayed fluorescence (Figure 1b).

As a subset of circadian transcription in the chloroplast is driven by regular oscillations of nuclear gene expression (Noordally *et al.* 2013) we assayed F_q'/F_m' in previously described circadian mutants (Figure 1c-h) under 50 μmol m^{-2} s^{-1} constant blue light to determine whether these rhythms of F_q'/F_m' were controlled by the nuclear circadian system. *toc1-4* is a null *toc1* allele with a short circadian period (Hazen *et al.* 2005a, Jones and Harmer 2011), *prr7-3* seedlings have a long circadian phenotype whereas seedlings lacking *LUX* are unable to maintain transcriptional circadian oscillations (Hazen*, et al.* 2005b). In agreement with these previous reports, *toc1-4* seedlings displayed a shorter circadian period of F_q'/F_m' with *toc1-4* seedlings having rhythms of 18.97 ± 0.07 hrs compared to 23.44 ± 0.12 hrs in wild type plants ($p<0.001$, Figure 1c and 1d). *prr7-3* seedlings displayed a longer circadian period of 25.70 ± 0.16 hours compared to 23.55 ± 0.10 hrs in wild type ($p<0.001$, Figures 1e and 1f). Hazen *et al.* previously reported that *lux*-2 seedlings retain a residual nuclear rhythmicity for the first 24 hours after

transfer to constant conditions (Hazen*, et al.* 2005b) and we observed a comparable phenotype when using chlorophyll fluorescence. F_q'/F_m' increases in *lux-2* seedlings for the first twelve hours before becoming arrhythmic at subjective dusk (Figure 1g and 1h). Such data suggest that F_q'/F_m' rhythms are strongly influenced by transcriptional rhythms previously documented in the nucleus.

F_q'/F_m' can be affected by multiple physiological parameters including the leaf's internal CO_2 concentration. As stomatal opening (which permits gas exchange between the leaf and atmosphere) is regulated by the circadian system we were curious how stomatal conductance varied over the course of our experimental conditions. We found that stomatal conductance continued to have a circadian rhythm under constant blue light, but that the peak of this activity was during the subjective morning, several hours after our observed peak of F_q'/F_m' (Figure 1i).

Phototropins are necessary to maintain the amplitude of circadian rhythms of F_q'/F_m' under dim blue light

Light input into the nuclear circadian system occurs via phytochromes, cryptochromes and the ZTL family of proteins, but a role for the phototropin blue light receptors has yet to be defined (Fankhauser and Staiger 2002, Christie *et al.* 2014, Hsu and Harmer 2014). As phototropins have recently been reported to relocalize to the surface of the chloroplast following blue light illumination (Kong *et al.* 2012) we assessed whether phototropins were necessary for circadian rhythms of F_q'/F_m' under 20 μmol m^{-2} s^{-1} constant blue light. While wild type seedlings maintained a rhythm of 24.79 ± 0.20 hrs, the rhythms observed in *p1p2* seedlings were dampened after three days of free run (Figure 2a-b). This dampening led to a significant increase in Relative Amplitude Error (RAE, Plautz*, et al.* 1997) in the rhythms of *p1p2* seedlings compared to wild type ($p<0.001$, Dunnett's test, Figure 2b). This loss of rhythmicity was not observed in *phot1-5* or *phot2-1* seedlings (Figure 2a-b). Despite the loss of amplitude of F_q'/F_m' rhythms we observed that rhythms of delayed fluorescence were maintained in *p1p2* seedlings (Figure 2c-d, Figure S2),

suggesting that phototropins are only influencing a subset of the processes regulated by the clock in the chloroplast. In addition, the role of phototropins in altering F_q'/F_m' was restricted to dim blue light- *p1p2* seedlings maintained rhythmic amplitude when transferred to 50 μmol m^{-2} s^{-1} rather than 20 μmol m^{-2} s^{-1} blue light (Figure 2e-f). Such data suggest that both phot1 and phot2 are required for the maintenance of F_q'/F_m' rhythms under 20 μmol m^{-2} s^{-1} blue light.

As F_q'/F_m' rhythms are altered by the nuclear circadian system (Figures 1c-1h) we performed qRT-PCR to determine whether phototropins were necessary to maintain oscillations of nuclear gene expression (Figure 3). The phase and amplitude of *CCA1*, *LHY* and *PRR9* transcript accumulation remained unchanged in *phot1-5*, *phot2-1* and *p1p2* seedlings transferred to either 20 or 50 μmol m^{-2} s^{-1} constant blue light when compared to wild type, consistent with previous reports that the nuclear clock is intact in plants lacking phototropins (Figure 3A-C, Figure S3, Devlin and Kay 2001). As such it appears that the loss of amplitude observed in F_q'/F_m' is not dependent upon wholesale changes in nuclear gene expression but is instead limited to changes within the chloroplast.

Plants lacking phototropins display impaired F_q'/F_m' rhythms under dynamic light regimes

Phototropins permit plants to respond to directional light stimuli and demonstrate a relocalization from the plasma membrane to the cytoplasm, chloroplast membrane and other intra-cellular structures within three minutes of blue light irradiation (Liscum and Briggs 1995, Kagawa *et al.* 2001, Sakamoto and Briggs 2002, Kong *et al.* 2006, Kaiserli *et al.* 2009). Such data suggest that phototropins are able to regulate responses to dynamic light environments and we therefore adapted our existing protocol to monitor oscillations of F_q'/F_m' under fluctuating light conditions in *p1p2* plants. We measured F_q'/F_m' over several days under a light scheme of 50 μmol m^{-2} s^{-1} blue light incorporating a 10-minute dark interval once every hour (Figure 4). Although F_q'/F_m' rhythms continued in wild type plants we noted that the amplitude of rhythmic F_q'/F_m' in *p1p2* plants was half that observed in wild type (0.011±0.0013 vs. 0.0066±0.00088 for wild type and

p1p2 respectively, Figure 4a). These data suggest that phototropins influence circadian rhythms under dynamic light regimes by enhancing rhythmic amplitude.

The inclusion of a dark period into our protocol enabled deconvolution of F_q'/F_m' into the contributing quenching parameters as this short interval was sufficient to revert the leaf into a dark-adapted state following our dim light conditions (Figure 4b). F_q'/F_m' is calculated from the maximum operating efficiency of PSII at a given light intensity (termed F_v'/F_m') and the realized fraction of this potential that is used for photochemistry (F_q'/F_v'). We were able to monitor rhythms of F_v'/F_m' in wild type plants grown under blue light following entrainment to symmetrical diurnal cycles (Figure 4c-d). Rhythms had a period of 23.85±0.10 hrs and were robust, with an average RAE of 0.30 (Figures 4d). F_v'/F_m' rhythms were less apparent in *p1p2* plants, with significant variation of the period of each individual plant within the measured cohort. The standard deviation of F_v'/F_m' period estimates of *p1p2* plants was 1.867hrs compared to 0.702hrs in wild type (Figure 4d), which suggests that rhythms of F_v'/F_m' were less coordinated within the *p1p2* group than wild type. A more substantial defect between wild type and *p1p2* seedlings was observed when we examined rhythms of F_q'/F_v' (Figure 4e). Modest rhythms of F_q'/F_v' continued in wild type plants, with a period of 24.63±0.35 hrs (Figure 4e). By contrast, only 50% of *p1p2* seedlings returned a period estimate with a RAE < 0.6 (Figure 4f). Such data indicate that rhythms of photochemical quenching are impaired in *p1p2* seedlings.

NPH3 is not required for the maintenance of chlorophyll fluorescence rhythms under dim blue light

Phototropic responses mediated by phototropins require a BTB protein, NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), that interacts with CULLIN3 as a substrate adaptor in order to target phot1 for ubiquitination (Motchoulski and Liscum 1999, Roberts *et al.* 2011, Liscum *et al.* 2014). In order to examine whether NPH3 is also required for the maintenance of chlorophyll fluorescence rhythms in the chloroplast we assessed whether plants lacking NPH3 displayed similar phenotypes to *p1p2* mutants under either dim blue light or our fluctuating light conditions

(Figure 5). Rhythms of F_q'/F_m' were maintained in *nph3-1* plants under 20 µmol m^{-2} s^{-1} constant blue light compared to wild type (Figure 5a), with a period of 24.29±0.21 hrs compared to 24.11 ±0.14 hrs in the control plants (p=0.42, Figure 5b). We next determined whether rhythms of maximum operating efficiency of PSII were perturbed in *nph3-1* seedlings, as we had observed for *p1p2* plants (Figures 4c-d). We found that rhythms of F_v'/F_m' were indistinguishable between wild type and *nph3-1* seedlings, and that these rhythms of F_q'/F_v' were maintained (Figure 5c-d). Such data suggest that the role of NPH3 is dispensable for phototropin-mediated rhythms of maximum PSII operating efficiency.

Discussion

The photosynthetic efficiency of PSII varies with a circadian rhythm

The adoption of delayed chlorophyll fluorescence (DF) methods has permitted the characterization of the circadian system in a wide range of species but the physiological and biochemical mechanisms underlying these rhythms remain elusive (Gould, *et al.* 2009, Dodd, *et al.* 2014). In an effort to improve understanding of these rhythms we used an alternative suite of methods using Chlorophyll a Fluorescence (CaF) to explore the role of the circadian system as a regulator of photosynthetic efficiency. Although F_q'/F_m' is a ratiometric measurement (and therefore is not directly affected by chloroplast movement, Brugnoli and Björkman 1992) we were concerned that leaf movement over the course of our experiment might produce shading artefacts that could be erroneously interpreted as circadian rhythms. To mitigate against this possibility we restrained leaf movement with a fine wire mesh (Figure S1). Rhythms of F_q'/F_m' continued when plants leaves were restrained in this way, confirming that these oscillations are indicative of subcellular processes rather than subtle changes in the light environment.

Our data indicate that the operating efficiency of PSII (F_q'/F_m') varied over circadian time under constant blue light (Figures 1 and 2) and demonstrate that F_q'/F_m' is a robust circadian output that peaks shortly before dawn, at least under constant conditions (Figure 1 and 2a-b).

Although we do not report on the molecular mechanism underlying these daily changes it is possible to speculate that the components of the photosynthetic apparatus vary over the course of the day to maximise energy absorption whilst limiting damage caused by excessive light harvesting, or that feedback mechanisms from the daily production of starch may induce alterations in the use of light for photochemistry (Dodd *et al.* 2015).

F_q'/F_m' can be influenced by many factors including stomatal conductance (which alters internal leaf CO_2 concentration) and CO_2 assimilation (Baker 2008). Rhythms of F_q'/F_m' have previously been reported in individual *Kalenkoë daigremontana* leaves, although in this case F_q'/F_m' peaked at subjective dusk (Wyka *et al.* 2005). This discrepancy most likely arises from the differing photochemistries of Arabidopsis and *K. daigremontana* as *K. daigremontana* completes crassulacean acid metabolism (CAM) and therefore temporally separates CO_2 harvesting from the Calvin cycle. The phase of F_q'/F_m' in Arabidopsis precedes stomatal opening as we observed that stomatal conductance peaked during the subjective morning rather than before dawn (Figure 1i), which is consistent with previous reports (Hennessey and Field 1991, Dodd, *et al.* 2004). Such data suggest that the observed F_q'/F_m' rhythms in Arabidopsis are not directly linked to stomatal opening although it remains possible that rhythmic stomatal opening varies CO_2 availability and subsequently contributes to changes in F_q'/F_m' during the subjective day by altering the rate of photochemical quenching.

Transcriptional oscillations in the nucleus regulate rhythms of photosynthetic efficiency

Well-defined transcriptional feedback loops regulate expression of approximately one third of the genome in Arabidopsis, subsequently influencing many downstream biological processes (Covington *et al.* 2008, Hsu and Harmer 2014). Our data indicate that the nuclear clock is required for rhythms of F_q'/F_m' within the chloroplast (Figure 1). We observed short period phenotypes in *toc1-4* (Figure 1c-d), and a long period phenotype in *prr7-3* (Figure 1e-f) while we were unable to detect rhythms in *lux-2* mutants (Figure 1g-h). Recent work by Noordally *et al.*

(2013) has revealed that a nuclear-encoded sigma factor, SIG5, is required to coordinate rhythms of gene expression between the nucleus and a subset of chloroplast genes although rhythms of DF were maintained in *sig5* seedlings (Noordally*, et al.* 2013). As the relationship between DF and CaF measurements has yet to be determined it will be of interest to evaluate whether *sig5* plants maintain F_q'/F_m' rhythms in addition to DF, or whether this mutant background would allow these alternate imaging methods to be distinguished.

Phototropins maintain circadian rhythms of F_q'/F_m' under low light or dynamic light conditions

Phytochromes, cryptochromes and the ZTL family each contribute to light perception by the nuclear circadian clock (Somers*, et al.* 1998, Devlin and Kay 2000, Baudry*, et al.* 2010, Pudasaini and Zoltowski 2013) but anecdotal reports have suggested that phototropins do not influence this aspect of the circadian system (Devlin and Kay 2001). To confirm these reports we monitored accumulation of *CCA1*, *LHY*, and *PRR9* transcripts under our experimental conditions. Accumulation of these transcripts appeared to be unaffected (Figures 3a-c), in line with a recent comprehensive analysis of GFP-tagged phototropins *in vivo* that did not identify a direct role for phototropins within the nucleus (Kong *et al.* 2013).

Phot1 and phot2 have recently been reported to localize to the surface of chloroplasts upon illumination with blue light as part of the well-characterized chloroplast avoidance and accumulation responses (Kong and Wada 2011, Kong*, et al.* 2013). We were therefore curious whether phototropins were necessary for circadian rhythms of F_q'/F_m' within these photosynthetic organelles. Under 20 μmol m^{-2} s^{-1} constant blue light we observed that *p1p2* mutants have a reduced amplitude of F_q'/F_m' rhythms, with these rhythms gradually dampening to apparent arrhythmia during the first four days of free-run (Figure 2a). It appears that both phot1 and phot2 contribute to this phenotype as neither single mutant displayed this phenotype (Figures 2a-b). Experiments using delayed fluorescence indicated a trend for longer circadian period in *phot1-5,*

phot2-1 and *p1p2* seedlings that was not apparent in F_q'/F_m' data, although these differences were not statistically significant (Figure 2d). These discrepancies between phenotypes reported by F_q'/F_m' and DF rhythms may indicate different underlying biological processes, and it will be of interest to further explore these mechanisms in the future. Such investigations will determine whether phototropins act to alter the constitution of the light harvesting complexes or if their role in maintaining robust circadian rhythms is an indirect consequence of either impaired chloroplast movement or stomatal conductance in *p1p2* plants.

F_q'/F_m' is mathematically derived from two quenching parameters calculated from chlorophyll fluorescence measurements, F_v'/F_m' and F_q'/F_v' (Baker 2008). These factors can be used to infer the physiological processes underlying these rhythms; changes in F_q'/F_v' indicate changes in processes related to photochemistry whereas fluctuations in F_v'/F_m' suggest that the light harvesting apparatus itself undergoes reorganization to facilitate changes in non-photochemical quenching (Baker 2008). Our data suggest that both these parameters contribute towards rhythms of F_q'/F_m' (Figure 4). Rhythms of F_v'/F_m' peaked approximately two hours after that of F_q'/F_v' in wild type (Figures 4c and 4e), which suggests that the optimal configuration of proteins associated with photosynthetic photochemistry and holoproteins comprising the light harvesting complex are not completely synchronized under constant conditions. One explanation for this delay could be that limits in F_q'/F_v' may lead to increased nonphotochemical quenching and the consequential rearrangement of the light harvesting apparatus. Interestingly, we found that *p1p2* plants were less able to coordinate circadian rhythms of F_v'/F_m' and were essentially arrhythmic with regards F_q'/F_v' (Figures 4c-f).

Although the phototropism signalling cascade initiated by phots requires NPH3 our data suggest that NPH3 is not required for the maintenance of circadian rhythms of F_q'/F_m' or F_q'/F_v' (Figure 5, Motchoulski and Liscum 1999). These data are in agreement with previous studies that demonstrated that NPH3 is not required for the initial phot1-mediated inhibition of hypocotyl growth, chloroplast accumulation response or for blue light-mediated stomatal opening (Folta and

Spalding 2001, Inoue *et al.* 2008). Instead, it remains possible that either phots relocalized to the chloroplast outer membrane initiate a signalling cascade or that cytoplasmic signalling intermediates other than NPH3 are required for signal transmission. Although we do not describe a mechanism for phototropin-initiated signalling across chloroplast membranes it is plausible that phot-interacting partners at the outer chloroplast membrane may allow coordination of the photosynthetic apparatus via this blue light sensor.

Rhythms of photosynthetic efficiency appear distinct from previously reported rhythms in the chloroplast

2-cysteine peroxiredoxins (2-CysPrx) are scavengers of reactive oxygen species within the chloroplast (Muthuramalingam *et al.* 2009) and recent reports have identified transcription-independent circadian oscillations of 2-CysPrx oxidation in Arabidopsis and *Ostreococcus tauri* (O'Neill *et al.* 2011, Edgar*, et al.* 2012). The localization of 2-CysPrx within the chloroplast is altered depending upon its oxidation status, forming multimers and associating with the thylakoid membrane upon oxidation (König *et al.* 2002, König *et al.* 2003). This altered localization increases the affinity of 2Cys-Prx for components of photosystem II (Muthuramalingam*, et al.* 2009) and reports from various plant models have reported that interaction with 2-CysPrx modulates enzyme activity (Caporaletti *et al.* 2007). These data suggest the hypothesis that circadian 2-CysPrx oxidation and subsequent interaction with photosystem II could alter photosynthetic parameters modulate photosynthetic efficiency. However plants lacking chloroplastic 2-CysPrxs did not display a significant difference in dark-adapted maximum photosynthetic efficiency, indicating that there is significant redundancy within the ROS scavenging system (Pulido *et al.* 2010). Given this reported redundancy and the discrepancy between the requirement for nuclear transcriptional control between our reported F_q'/F_m' rhythms and 2-CysPrx oxidation it instead appears that these processes oscillate independently of one

another. Further work will be required to fully explore this possibility but it is apparent that the circadian system within the chloroplast has numerous contributing factors.

We look forward to future developments that exploit chlorophyll fluorescence techniques to monitor circadian rhythms in PSII photosynthetic efficiency. Use of this technology will enable the measurement of circadian rhythms in numerous photosynthetic species and improve our understanding of how photochemical activities within the chloroplast are regulated by light signalling and circadian signals from the nucleus.

Experimental Procedures

Plant Material and Growth Conditions

nph3-1, phot1-5, *phot2-1* and *phot1-5 phot2-1* double mutant seed have been previously described (Liscum and Briggs 1995, Motchoulski and Liscum 1999, Jarillo *et al.* 2001, Kagawa*, et al.* 2001, Sakai *et al.* 2001), as have *lux-2, toc1-4* and *prr7-3* (Farré *et al.* 2005, Hazen*, et al.* 2005a, Hazen*, et al.* 2005b). Plants were grown under cool fluorescent white light under a 12/12 photoperiod at 60 μmol m^{-2} s^{-1} in A1000 Adaptis chambers (Conviron Europe Ltd, Isleham, UK) for 6-12 days before transfer to experimental conditions outlined below.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence parameters were recorded with a Fluorimager imaging system using automated camera control and image processing scripts provided by the manufacturer (Technologica Ltd, Colchester, UK). Approximately 30 individually spaced seedlings were entrained for 12 days in 12:12 light:dark cycles on half-strength Murashige and Skoog (MS) media without supplemental sucrose for 12 days before transfer to the imaging chamber. After transfer from the growth chamber plants were illuminated with either 20 or 50 μmol m^{-2} s^{-1} blue light using blue LEDs, with measuring pulses of 5713 μmol m^{-2} s^{-1} blue light for 800 ms once per hour. Chlorophyll fluorescence was imaged using a Dolphin camera (Allied Vision Technologies,

UK) through a longpass filter to exclude the blue light from the LEDs. Images of chlorophyll fluorescence emission from light-adapted leaves (*F'*) and maximal fluorescence emission from the light-adapted leaf following the saturating measuring pulse (F_m') were used to calculate F_q'/F_m' where $F_q' = F_m' - F'$ (Baker 2008). Measurement of F_q'/F_v' and F_v'/F_m' necessitated the inclusion of a dark adaptation step for 10 minutes before measurement to allow calculation of the minimal fluorescence from a light-adapted leaf (F_o') where $F_o' = F_o/[(F_v/F_m) + (F_o/F_m)]$ (Baker 2008). Patterns of F_q'/F_m' were fitted to cosine waves using Fourier Fast Transform-Non-Linear Least Squares (Plautz*, et al.* 1997) to estimate circadian period length and additional circadian parameters.

Luciferase and Delayed fluorescence imaging

To complete luciferase imaging individual seedlings were entrained for 6 days in 12:12 light:dark cycles under white light on half-strength Murashige and Skoog (MS) media without supplemental sucrose before being sprayed with 3 mM D-luciferin in 0.01% Triton X-100. Plants were then transferred to free-running conditions under 20 μmol m^{-2} s^{-1} blue light provided by blue LEDs (peak emission at 459nm), with images being captured every two hours (Jones *et al.* 2010). For delayed fluorescence imaging groups of 15-20 seedlings were entrained for 12 days on half-strength MS media without supplemental sucrose before transfer to free-running conditions under 20 μmol m^{-2} s^{-1} blue light, with images being captured every hour (Gould*, et al.* 2009). Imaging was completed over 5 days using either a Photek HRPCS5 system or an Andor iKon-M CCD camera controlled by μManager (Edelstein *et al.* 2010) before data was processed using ImageJ (Schneider *et al.* 2012). Patterns of luciferase activity or delayed fluorescence were fitted to cosine waves using Fourier Fast Transform-Non-Linear Least Squares (FFT-NLLS, Plautz*, et al.* 1997) to estimate circadian period length. RAE is a measure of rhythmic robustness, with a value of 0 indicating an exact fit to a cosine wave (Plautz et al., 1997).

qRT-PCR

Following entrainment, plants were transferred to 20 or 50 μmol m^{-2} s^{-1} blue light (458 nm peak emission) provided by light emitting diodes (PowerPax UK Ltd, Theale, UK). Tissue was harvested at the indicated time before RNA was isolated from 10-15 seedlings for each data point using Tri Reagent® according to the manufacturer's protocol (Sigma Aldrich, Dorset, UK). Reverse transcription was performed using RevertAid reverse transcriptase following DNAse treatment (Fisher Scientific, Loughborough, UK). qRT-PCR was performed using a BioRad CFX96 Real-Time system. 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 Table S1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *CCA1*, At2g46830; *GI*, At1g22770; *LHY*, At1g01060; *LUX,* At3g46640; *NPH3*, At5g64330*; PP2A*, At1g13320; *PHOT1,* At3g45780; *PHOT2*, At5g58140; *PRR7*, At5g02810; *PRR9*, At2g46790 and *TOC1*, At5g61380.

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Short Legends for Supplementary Information

Figure S1. F_q'/F_m' rhythms continue in the absence of leaf movement.

Figure S2. Circadian rhythms of delayed fluorescence in Arabidopsis seedlings under constant blue light.

Figure S3. Expression of circadian clock-regulated genes in *p1p2* seedlings under 50 μmol m^{-2} s^{-1} constant blue light.

Table S1. Oligos used in this study.

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Figure Legends

Figure 1. PSII operating efficiency varies over circadian time. (a) Circadian period estimates of wild-type Columbia seedlings plotted against Relative Amplitude Error (RAE) under 20 µmol m^{-2} s^{-1} constant blue light using luciferase imaging (*CCA1::LUC2*), delayed fluorescence or PSII operating efficiency (F_q'/F_m'). Plants were grown on MS media for 6 days (luciferase imaging) or 12 days (delayed fluorescence and F_q'/F_m') before imaging. RAE is a measure of rhythmic robustness, with a value of 0 indicating an exact fit to a cosine wave (Plautz et al., 1997). Standard error of the mean is shown, n=19-28. Data from one of three independent experiments are shown. **(b)** Circadian phase of data presented in (a). **(c, e, g)** Measurements of F_q'/F_m' in *toc1-4* (c), *prr7-3* (e) and *lux-2* (g) seedlings plotted against Columbia under constant blue light. Seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods on MS media for 12 days before being transferred to 50 µmol m^{-2} s^{-1} constant blue light. Data from one of three independent experiments are shown and are mean values of multiple seedlings (n=11-20). Standard error of the mean is presented every 10 hours for clarity. **(d, f, h)** Period estimates of F_q'/F_m' circadian rhythms in *toc1-4* (d), *prr7-3* (f) and *lux-2* (h) from data presented in (c), (e) and (g). Asterices indicate $p<0.001$ compared to respective Columbia control (Student's t test). **(i)** Stomatal conductance of Arabidopsis seedlings under constant blue light. Columbia plants were grown on soil under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 21 days before being transferred to 50 µmol m^{-2} s^{-1} constant blue light. Stomatal conductance was recorded every 3 hours. Error bars indicate standard deviation, n=6. Data from one of two independent experiments are shown.

Figure 2. Phototropins maintain circadian rhythms of F_q'/F_m' under dim blue light. (a) F_q'/F_m' rhythms in Columbia (black), *phot1-5* (red), *phot2-1* (purple) and *phot1-5 phot2-1* (*p1p2*,

blue) seedlings. Seedlings were grown under 60 μmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods on MS media for 12 days before being imaged under 20 μmol m^{-2} s^{-1} constant blue light. Error bars represent standard error of the mean and are presented every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(b)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using F_q'/F_m'. Data were pooled from three independent experiments, n=20-23. * indicates a significant difference in RAE compared to wild type ($p<0.001$, Dunnett's test). **(c)** Circadian rhythms of delayed fluorescence in Columbia, *phot1-5*, *phot2-1* and *p1p2*. Seedlings were treated as described in (a). Averaged data from three independent experiments are shown, n=23-26. **(d)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using delayed fluorescence. Data are replotted from (c). **(e)** Measurements of F_q'/F_m' rhythms in Arabidopsis seedlings under 50 μmol m^{-2} s^{-1} constant blue light. Seedlings were treated as described in (a) before transfer to constant blue light with this higher fluence rate. Error bars represent standard error of the mean and are presented every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(f)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using F_q'/F_m'. Averaged data from two independent experiments are shown, n=12-16.

Figure 3. Expression of circadian clock-regulated genes in 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 *CCA1* **(a)**, *LHY* **(b)**, and *PRR9* **(c)** mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d on MS media before being moved to constant conditions with 20 μmol m^{-2} s^{-1} blue light. Data for each gene were compared with an internal control (PP2a) before being normalized to the peak of wild-type expression. Data are the average of three biological replicates, error bars show standard error of the mean.

Figure 4. Rhythms of photosynthetic operating parameters in Arabidopsis seedlings under fluctuating light. (a) Measurements of F_q'/F_m' in Columbia and *phot1-5 phot2-1* (*p1p2*) seedlings under fluctuating 50 μmol m^{-2} s^{-1} blue light incorporating 10 minute intervals for dark adaptation every hour. Seedlings were grown under 60 μmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days on MS media before being imaged under this light regime. Standard error of the mean is shown every 10 hours for clarity, n=14-19. Data from one of three independent experiments are shown. **(b)** Dark adaptation of Columbia and *p1p2* seedlings following blue light irradiation. Seedlings were initially held in constant darkness for 1 hour before F_v/F_m was calculated. Plants were then illuminated with 50 μmol m^{-2} s^{-1} blue light for one hour before being transferred to darkness for the indicated intervals. F_v/F_m was measured at the indicated time after transfer back to darkness (min). Error bars indicate standard error of the mean, n=19. **(c)** Measurements of F_v'/F_m' in Arabidopsis seedlings over circadian time. Columbia (Col) and *p1p2* seedlings were grown under 60 μmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days before being imaged under 50 μmol m^{-2} s^{-1} fluctuating blue light. Error bars indicate standard error of the mean, n=14-19. Data from one of three independent experiments are shown. **(d)** Period estimates of F_v'/F_m' circadian rhythms plotted against Relative Amplitude Error. Error bars show standard error of the mean, n=34-46. Averaged data from three independent experiments are shown. Plants were treated as described in (a). **(e)** Measurements of F_q'/F_v' in Arabidopsis seedlings over circadian time. Wild type and *p1p2* seedlings were treated as described in (a). Error bars indicate standard error of the mean, n=14-19. Data from one of three independent experiments are shown. **(f)** Proportion of seedlings returning an F_q'/F_v' rhythm estimate with an RAE<0.6. Plants were treated as described in (a). Percentages shown are the average of three independent experiments. * indicates P<0.01, Student's t-test.

Figure 5. Rhythms of photosynthetic operating parameters in *nph3* seedlings. (a) Measurements of F_q'/F_m' in Columbia (black), *nph3-1* (green), and *phot1-5 phot2-1* (*p1p2*, dotted

blue) seedlings under 20 μmol m^{-2} s^{-1} constant blue light. Seedlings were grown under 60 μmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days on MS media before being transferred to constant light**.** Standard error of the mean is shown every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(b)** Circadian period estimates of F_q'/F_m' in Columbia, *p1p2* and *nph3-1* seedlings under 20 μmol m^{-2} s^{-1} constant blue light. Error bars indicate standard error of the mean, n=19-26. Averaged data from three independent experiments are shown. **(c)** Measurements of F_q'/F_v' in Arabidopsis seedlings over circadian time. Columbia, *nph3-1* and *p1p2* seedlings were grown under 60 μmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days before being imaged under 50 μmol m^{-2} s^{-1} fluctuating blue light. Standard error of the mean is shown every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(d)** Circadian period estimates of F_q'/F_v' in Columbia, *p1p2* and *nph3-1* seedlings under 50 μmol m^{-2} s^{-1} fluctuating blue light. Data are the average of two independent experiments, n=13-23.

Figure 1

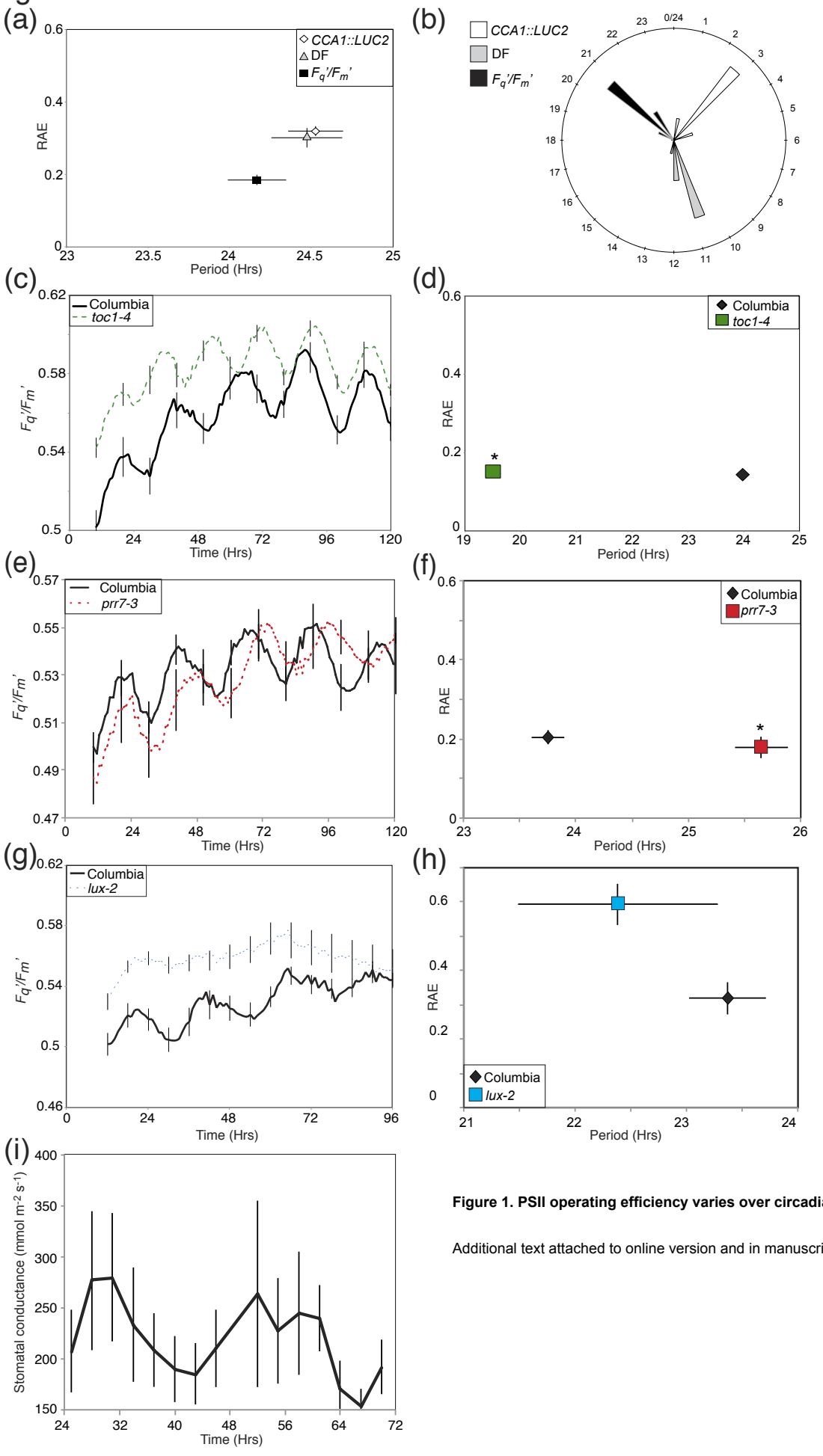

Figure 1. PSII operating efficiency varies over circadian time.

Additional text attached to online version and in manuscript text.

Figure 2

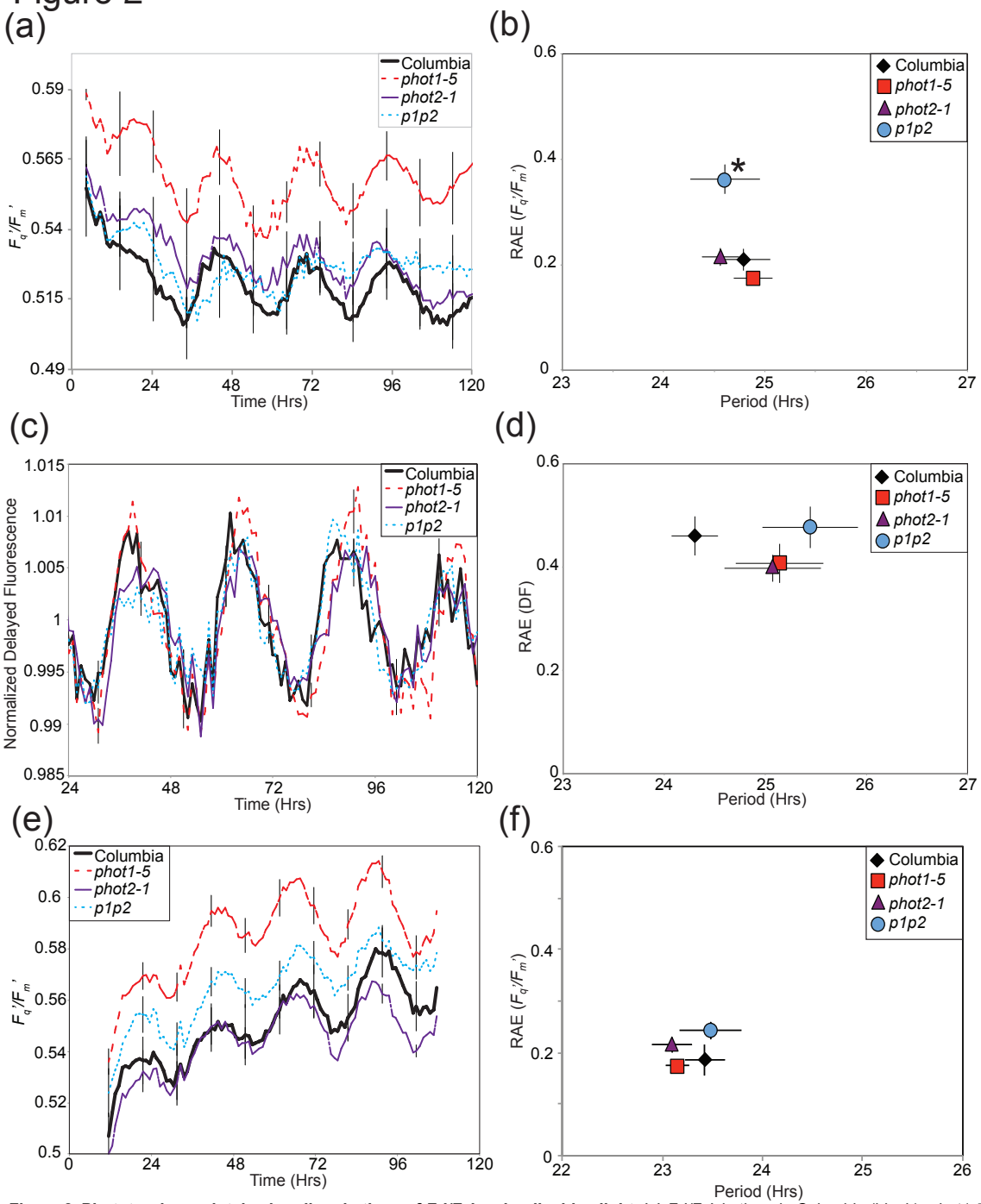

Figure 2. Phototropins maintain circadian rhythms of F_q'/F_m' under dim blue light. (a) F_q'/F_m' rhythms in Columbia (black), *phot1-5* (red), *phot2-1* (purple) and *phot1-5 phot2-1* (*p1p2*, blue) seedlings. Seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods on MS media for 12 days before being imaged under 20 µmol m^{-2} s^{-1} constant blue light. Error bars represent standard error of the mean and are presented every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(b)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using F_q'/F_m'. Data were pooled from three independent experiments, n=20-23. * indicates a significant difference in RAE compared to wild type ($p<0.001$, Dunnett's test). **(c)** Circadian rhythms of delayed fluorescence in Columbia, *phot1-5*, *phot2-1* and *p1p2*. Seedlings were treated as described in (a). Averaged data from three independent experiments are shown, n=23-26. **(d)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using delayed fluorescence. Data are replotted from (c). **(e)** Measurements of F_q'/F_m' rhythms in Arabidopsis seedlings under 50 µmol m^{-2} s^{-1} constant blue light. Seedlings were treated as described in (a) before transfer to constant blue light with this higher fluence rate. Error bars represent standard error of the mean and are presented every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(f)** Circadian period estimates of seedlings plotted against Relative Amplitude Error (RAE) using F_q'/F_m'. Averaged data from two independent experiments are shown, n=12-16.

Figure 3

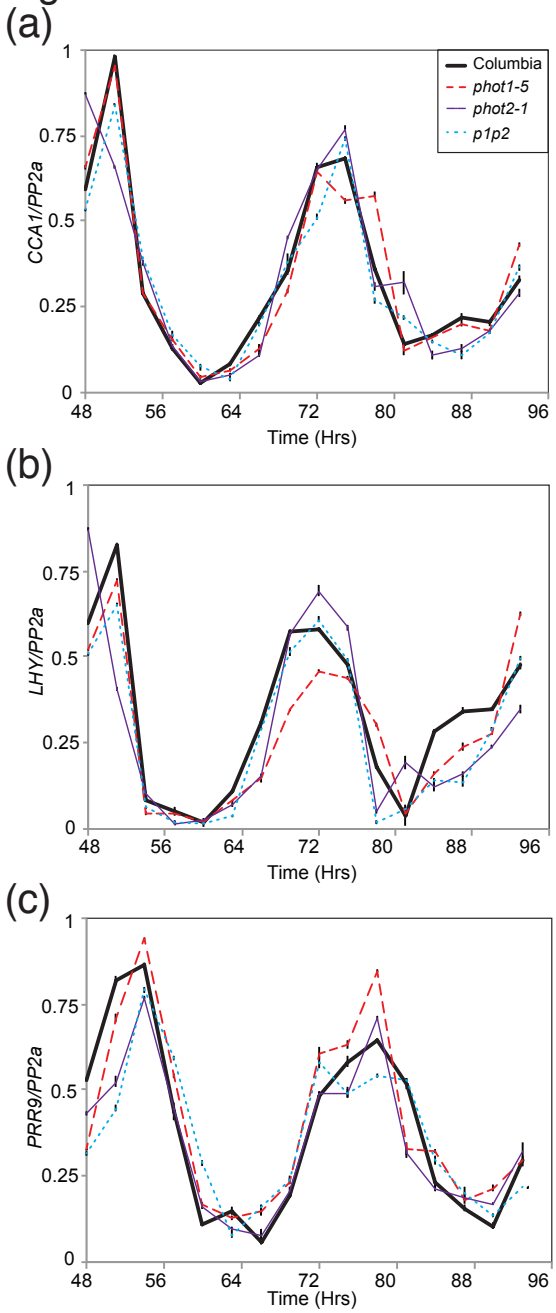

Figure 3. Expression of circadian clock-regulated genes in under constant blue light. Transcript accumulation in wild type (Columbia, solid black), *phot1-5* (dashed red), *phot2-1* (purple) and *phot1-5 phot2-1* (*p1p2,* dotted blue) mutants was compared using qRT-PCR. Levels of *CCA1* (a), *LHY* (b), and *PRR9* (c) mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d on MS media before being moved to constant conditions with 20 µmol m^{-2} s^{-1} blue light. Data for each gene were compared with an internal control (*PP2a*) before being normalized to the peak of wild-type expression. Data are the average of three biological replicates, error bars show standard error of the mean.

Figure 4

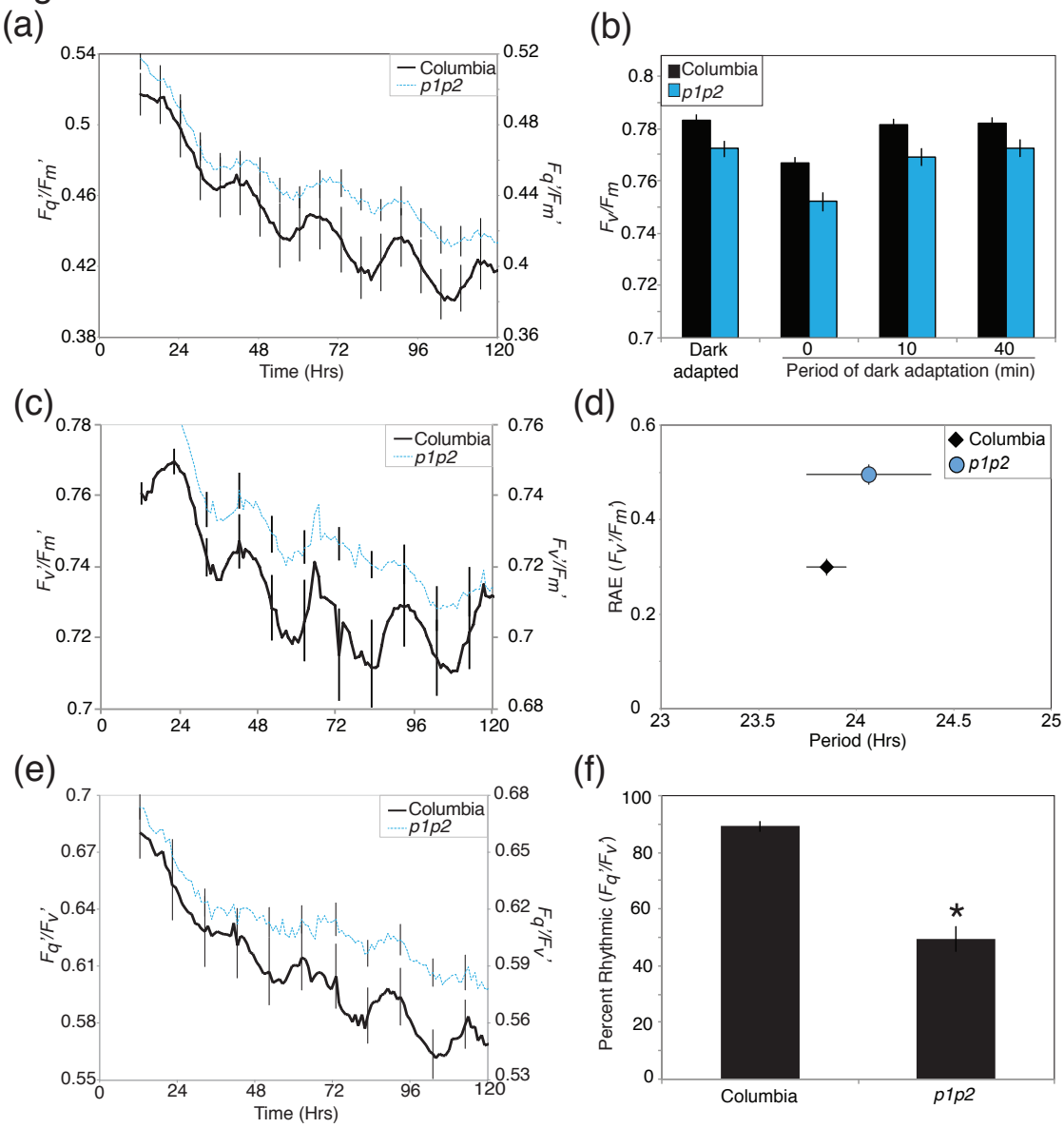

Figure 4. Rhythms of photosynthetic operating parameters in Arabidopsis seedlings under fluctuating light. (a) Measurements of F_q'/F_m' in Columbia and *phot1-5 phot2-1* (*p1p2*) seedlings under fluctuating 50 µmol m^{-2} s^{-1} blue light incorporating 10 minute intervals for dark adaptation every hour. Seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days on MS media before being imaged under this light regime. Standard error of the mean is shown every 10 hours for clarity, n=14-19. Data from one of three independent experiments are shown. **(b)** Dark adaptation of Columbia and *p1p2* seedlings following blue light irradiation. Seedlings were initially held in constant darkness for 1 hour before F_v/F_m was calculated. Plants were then illuminated with 50 µmol m^{-2} s^{-1} blue light for one hour before being transferred to darkness for the indicated intervals. F_v/F_m was measured at the indicated time after transfer back to darkness (min). Error bars indicate standard error of the mean, n=19. **(c)** Measurements of F_v'/F_m' in Arabidopsis seedlings over circadian time. Columbia (Col) and *p1p2* seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days before being imaged under 50 µmol m^{-2} s^{-1} fluctuating blue light. Error bars indicate standard error of the mean, n=14-19. Data from one of three independent experiments are shown. **(d)** Period estimates of F_v'/F_m' circadian rhythms plotted against Relative Amplitude Error. Error bars show standard error of the mean, n=34-46. Averaged data from three independent experiments are shown. Plants were treated as described in (a). Error bars indicate standard error of the mean, n=14-19. Data from one of three independent experiments are shown. **(e)** Measurements of F_q'/F_v' in Arabidopsis seedlings over circadian time. Wild type and *p1p2* seedlings were treated as described in (a). **(f)** Proportion of seedlings returning an F_q'/F_v' rhythm estimate with an RAE<0.6. Plants were treated as described in (a). Percentages shown are the average of three independent experiments. * indicates P<0.01, Student's t-test.

Figure 5

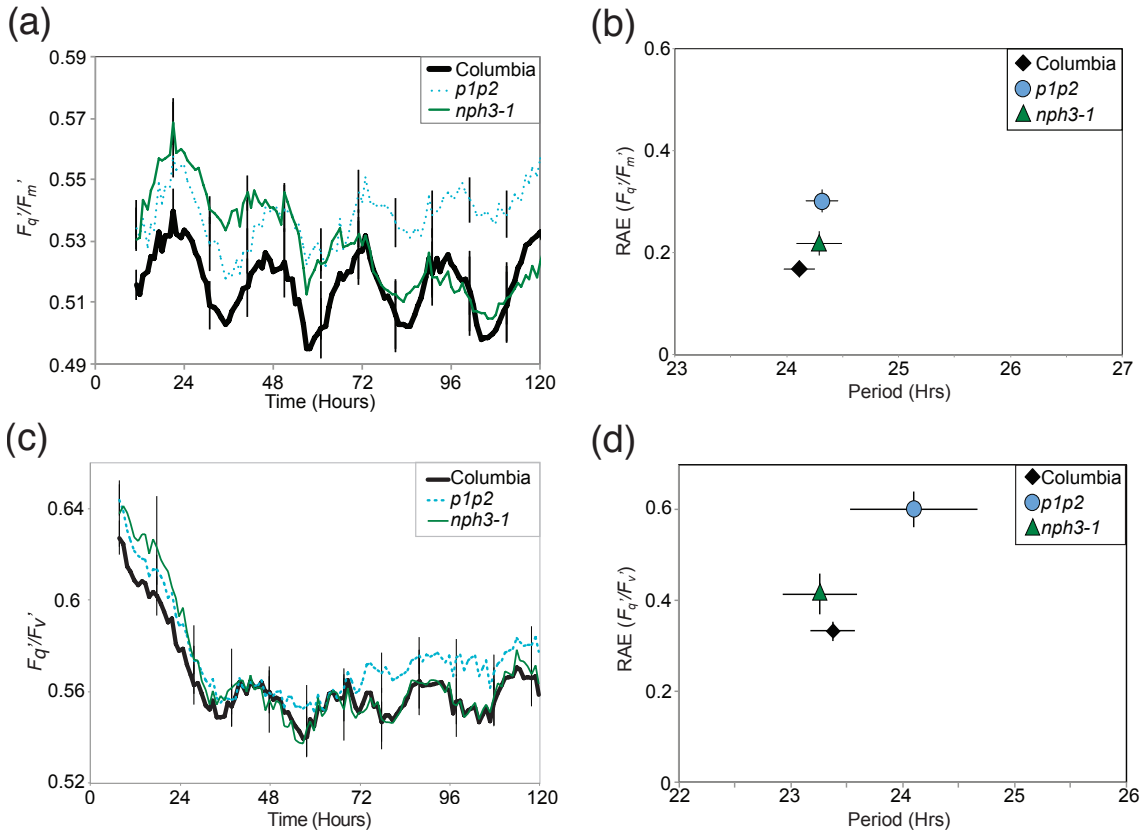

Figure 5. Rhythms of photosynthetic operating parameters in nph3 seedlings. (a) Measurements of F_q'/F_m' in Columbia (black), *nph3-1* (green), and *phot1-5 phot2-1* (*p1p2*, dotted blue) seedlings under 20 µmol m^{-2} s^{-1} constant blue light. Seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days on MS media before being transferred to constant light. Standard error of the mean is shown every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(b)** Circadian period estimates of F_q'/F_m' in Columbia, *p1p2* and *nph3-1* seedlings under 20 µmol m^{-2} s^{-1} constant blue light. Error bars indicate standard error of the mean, n=19-26. Averaged data from three independent experiments are shown. **(c)** Measurements of F_q'/F_v' in Arabidopsis seedlings over circadian time. Columbia, *nph3-1* and *p1p2* seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods for 12 days before being imaged under 50 µmol m^{-2} s^{-1} fluctuating blue light. Standard error of the mean is shown every 10 hours for clarity, n=7. Data from one of three independent experiments are shown. **(d)** Circadian period estimates of F_q'/F_v' in Columbia, *p1p2* and *nph3-1* seedlings under 50 µmol m^{-2} s^{-1} fluctuating blue light. Data are the average of two independent experiments, n=13-23.

Figure S1

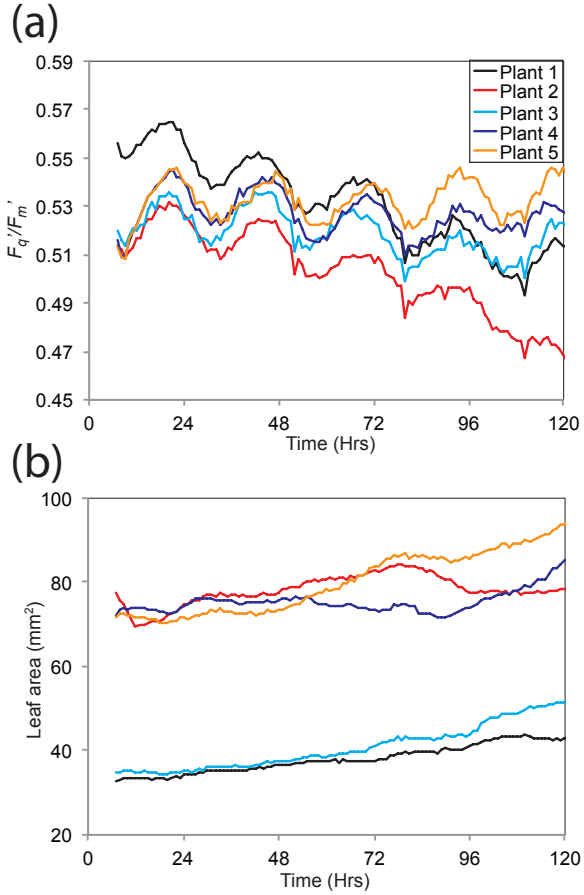

Figure S1. F_q'/F_m' rhythms continue in the absence of leaf movement. (a) Individual traces showing rhythms of F_q'/F_m' of Columbia seedlings restrained with a fine wire gauze. Plants were grown on soil for three weeks in 12:12 light:dark cycles before being restrained under 20 µmol m^{-2} s^{-1} constant blue light for 5 days, n=6. **(b)** Visible leaf area in plants described in (a).

Figure S2

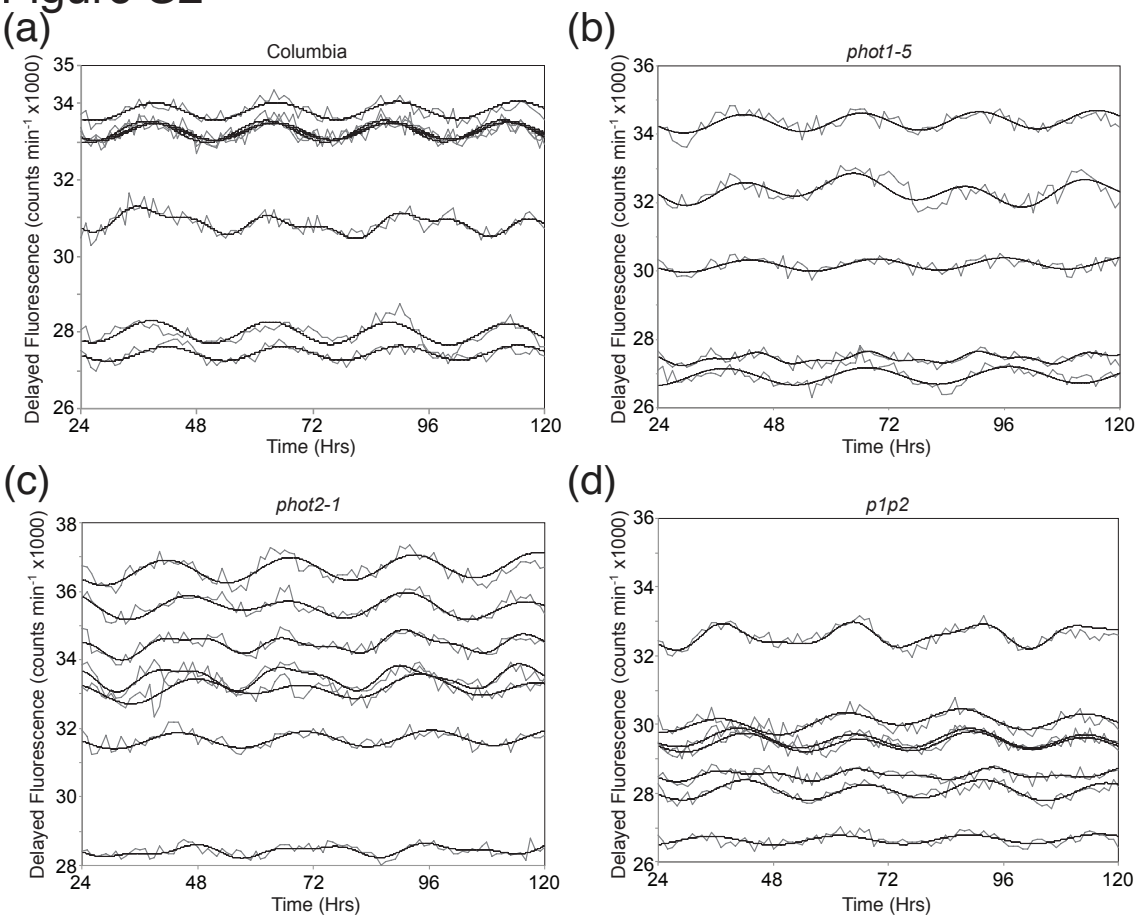

Figure S2. Circadian rhythms of delayed fluorescence in Arabidopsis seedlings under constant blue light. Traces for individual group of Columbia **(a)**, *phot1-5* **(b)**, *phot2-1* **(c)** and *phot1-5 phot2-1* (*p1p2,* **(d)**) seedlings are shown (grey), with overlaid cosine waves fitted by FFT-NLLS (black). Seedlings were grown under 60 µmol m^{-2} s^{-1} cool white light with 12:12 light:dark photoperiods on MS media for 12 days before being imaged under 20 µmol m^{-2} s^{-1} constant blue light. Data is presented from one of three independent experiments.

Figure S3

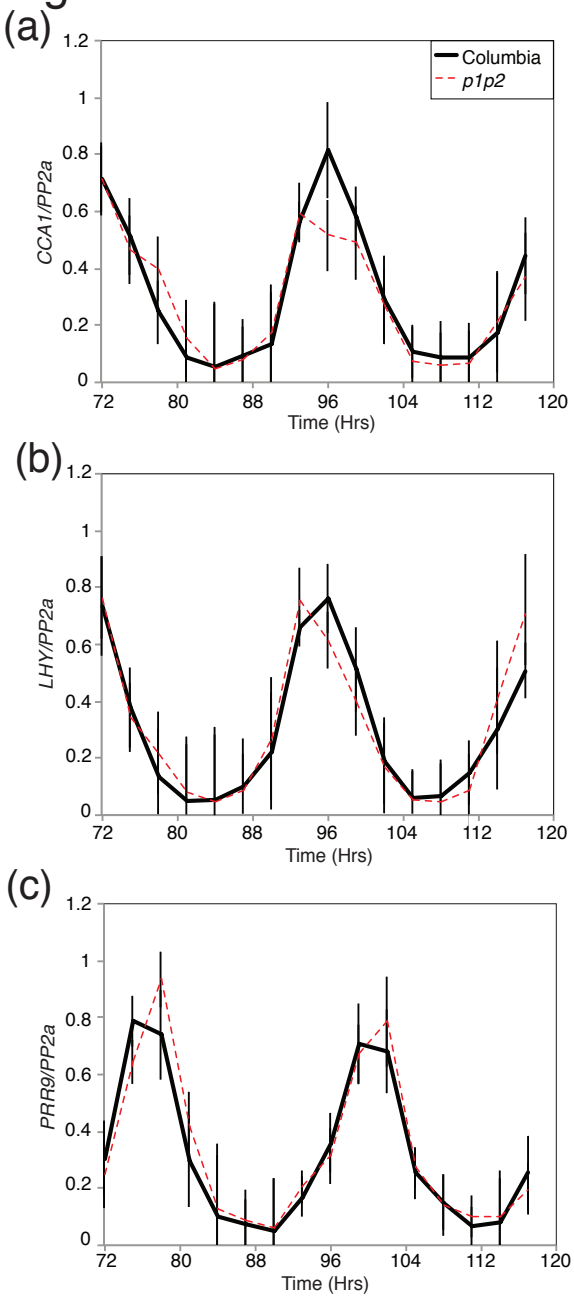

Figure S3. Expression of circadian clock-regulated genes in *p1p2* seedlings under 50 µmol m^{-2} s^{-1} constant blue light. Gene expression in wild type (Col, solid line) and *p1p2* (dashed red) mutants was compared using qRT-PCR. Levels of *CCA1* **(a)**, *LHY* **(b)**, and *PRR9* **(c)** mRNA were assessed. Plants were entrained to 12:12 LD cycles for 12 d before being moved to constant conditions with 50 µmol m^{-2} s^{-1} blue light. mRNA levels for each gene were normalized to *PP2a*. Data are the mean of three independent experiments; SEM is shown.

Name	Sequence	Reference
PP2a F	TAACGTGGCCAAAATGATGC	Czechowski *et al*. 2005
PP2a R	GTTCTCCACAACCGATTGGT	Czechowski *et al*. 2005
CCA1 F	CAGCTCCAATATAACCGATCCAT	Mockler *et al.* 2004
CCA1 R	CAATTCGACCCTCGTCAGACA	Mockler *et al.* 2004
PRR9 F	GTTGAAGAGGAAAGATCGATGCTT	Jones *et al.* 2012
PRR9 R	CTGCTCTGGTACCGAACCTTTT	Jones *et al.* 2012
LHY F	CAATGCAACTACTGATTCGTGGAA	Mockler *et al.* 2004
LHY R	GCTATACGACCCTCTTCGGAGAC	Mockler *et al.* 2004

Table S1. Oligos used in this study.