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Light inputs shape the Arabidopsis circadian system

Bénédicte Wenden[1], László Kozma-Bognár[1,†], Kieron D. Edwards[1,‡], Anthony J. W. Hall[2], James C. W. Locke[4] and Andrew J. Millar[1,3,*]

[1]*School of Biological Sciences, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, UK,*

[2]*School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK,*

[3]*Centre for Systems Biology at Edinburgh, C. H. Waddington Building, King's Buildings, Edinburgh EH9 3JD, UK, and*

[4]*Division of Biology and Department of Applied Physics, California Institute of Technology, M/C 114-96, Pasadena, CA 91125, USA*

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[*]For correspondence (fax +44 131 651 9068; e-mail andrew.millar@ed.ac.uk).

[†]Present address: Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary.

[‡]Present address: Advanced Technologies (Cambridge) Ltd, Cambridge, UK.

SUMMARY

The circadian clock is a fundamental feature of eukaryotic gene regulation that is emerging as an exemplar genetic sub-network for systems biology. The circadian system in Arabidopsis plants is complex, in part due to its phototransduction pathways, which are themselves under circadian control. We therefore analysed two simpler experimental systems. Etiolated seedlings entrained by temperature cycles showed circadian rhythms in the expression of genes that are important for the clock mechanism, but only a restricted set of downstream target genes were rhythmic in microarray assays. Clock control of phototransduction pathways remained robust across a range of light inputs, despite the arrhythmic transcription of light-signalling genes. Circadian interactions with light signalling were then analysed using a single active photoreceptor. Phytochrome A (phyA) is expected to be the only active photoreceptor that can mediate far-red (FR) light input to the circadian clock. Surprisingly, rhythmic gene expression was profoundly altered under constant FR light, in a phyA-dependent manner, resulting in high expression of evening genes and low expression of morning genes. Dark intervals were required to allow high-amplitude rhythms across the transcriptome. Clock genes involved in this response were identified by mutant analysis, showing that the *EARLY FLOWERING 4* gene is a likely target and mediator of the FR effects. Both experimental systems illustrate how profoundly the light input pathways affect the plant circadian clock, and provide strong experimental manipulations to understand critical steps in the plant clock mechanism.

Keywords: circadian rhythms, biological clocks, *Arabidopsis thaliana,* temperature, far red light, phytochrome, gene circuit.

INTRODUCTION

The circadian clock is a 24-h endogenous timer that allows the correct temporal regulation of physiological, biochemical and developmental processes. Recent studies have indicated that over 30% of the *Arabidopsis thaliana* (Arabidopsis) transcriptome is driven by the circadian clock (Harmer *et al.*, 2000; Michael *et al.*, 2008; Covington *et al.*, 2008), thus potentially regulating many metabolic pathways (Covington *et al.*, 2008). The mechanism of clocks in all organisms includes interlocked transcriptional–translational feedback loops. In Arabidopsis, a current model of these interlocked loops incorporates two closely related MYB transcription factors, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LONG ELONGATED HYPOCOTYL* (*LHY*), that inhibit the expression of evening-expressed genes such as a pseudo-response regulator *TIMING OF CAB EXPRESSION 1* (*TOC1*) (Alabadi *et al.*, 2001). TOC1 and other proteins activate (or de-repress) the expression of *CCA1* and *LHY* completing the loop. The expression of *CCA1* and *LHY* is also tightly regulated by other clock components, including sequential inhibition by PRR9, PRR7 and PRR5, which together form a morning loop (Farré *et al.*, 2005; Salome and McClung, 2005; Locke *et al.*, 2006; Pokhilko *et al.*, 2010). A further negative feedback between the evening-expressed *GIGANTEA* (*GI*) component and *TOC1* was proposed, and other components of this loop remain to be identified (Locke *et al.*, 2005).

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For the clock to be useful, the endogenous period must be synchronised (entrained) to match the 24-h environmental cycle (Johnson *et al.*, 2003; Hotta *et al.*, 2007). The strongest entrainment signals are temperature and light. While much has been studied about how organisms integrate light signals into the clock, how ambient thermocycles influence the clock remains mostly unknown. Alternative splicing has been implicated in *Neurospora crassa* and *Drosophila melanogaster* (Colot *et al.*, 2005; Diernfellner *et al.*, 2007; Low *et al.*, 2008). At least three families of photoreceptors have been identified as transducing light signals to reset the clock, the blue light sensing cryptochromes (*CRY1* and *CRY2*), the red/far-red light (R/FR) sensing phytochromes (*PHYA*, *PHYB*, *PHYD*, *PHYE*) (Somers *et al.*, 1998; Devlin and Kay, 2000; Yanovsky *et al.*, 2000) and a family of three F-box proteins, including *ZEITLUPE* (*ZTL*) (Imaizumi *et al.*, 2003; Kim *et al.*, 2007; Baudry *et al.*, 2010). These nine photoreceptors transduce light signals to clock genes and proteins (Fankhauser and Staiger, 2002; Nagy and Schafer, 2002; Kim *et al.*, 2007), with both specialised and overlapping roles.

Physiologically, light stable phytochromes (phyB to phyE) mediate low-fluence responses (LFR) which are R/FR reversible (Furuya and Schafer, 1996). In addition to LFR, the light-unstable phyA also participates in the very low-fluence responses (VLFR) which are activated by very low-intensity light of any visible wavelength and are not FR reversible (Botto *et al.*, 1996; Shinomura *et al.*, 1996), and the high-irradiance response (HIR) requiring long periods of strong light exposure (Furuya and Schafer, 1996; Marrocco *et al.*, 2006). Far-red light normally participates at several stages of plant morphogenesis including de-etiolation, where phyA is the principal photoreceptor involved in FR light perception (Yanovsky *et al.*, 1995; Botto *et al.*, 1996; Neff and Chory, 1998). Components of the phyA transduction pathway include *EMPFINDLICHER IM DUNKELROTEN LICHT 1* (*EID1*), an F-box protein that functions as a negative regulator (Marrocco *et al.*, 2006).

The chlorophyll *a/b*-binding protein (*CAB*, also known as *LHCB*) gene family includes some of the best-characterised phytochrome regulated genes in Arabidopsis, with gene activation mediated by VLFR, LFR and HIR phytochrome responses (Hamazato *et al.*, 1997). In addition to this light regulation, *CAB2* (*LHCB1.1*) expression is coupled to the circadian clock in both etiolated and light-grown seedlings (Millar and Kay, 1991; Millar *et al.*, 1992). In light-grown seedlings transferred to the dark (dark-adapted seedlings), acute induction of *CAB2* by light is modulated ('gated') by the clock. Brief light treatments during the subjective night induce much less *CAB* expression than those applied during the subjective day (Millar and Kay, 1996). In the *early flowering 3* (*elf3*), *early flowering 4* (*elf4*) and *time for coffee* (*tic*) mutants this gating of *CAB* induction is defective (McWatters *et al.*, 2000, 2007; Hall *et al.*, 2003). In *elf3* this defect may be due to the absence of a physical interaction between ELF3 protein and phyB (Liu *et al.*, 2001). *CAB* expression in etiolated seedlings can be induced by phyA and phyB (Anderson *et al.*, 1997), but past studies of circadian gating have used light treatments that target phyB responses.

Results that demonstrated the complexity due to active photoreceptors motivated us to analyse the clock in two physiologically simplified conditions. Where etiolated seedlings are temperature-entrained, we demonstrate that circadian regulation shares several properties with the clock of light-grown plants but controls fewer target genes. An alternative system used the response to FR light, governed primarily by phyA (Neff and Chory, 1998). We show dramatic effects on the circadian clock under constant FR conditions but not under far-red/dark (FR/D) cycles.

RESULTS

Circadian modulation of phytochrome signalling

The clock has been shown to regulate the expression of *PHY* transcripts to varying degrees (Kozma-Bognar *et al.*, 1999; Hall *et al.*, 2001; Toth *et al.*, 2001). It was unclear whether this transcriptional control was required for the circadian modulation of phytochrome responses (gating). Transgenic lines over-expressing *PHYB* from the cauliflower mosaic virus 35S promoter (*35S:PHYB*) in the *CAB:LUC* background allowed us to test this. Dark-adapted *35S:PHYB* plants maintained a higher basal level of *CAB:LUC* expression (Figure 1), but

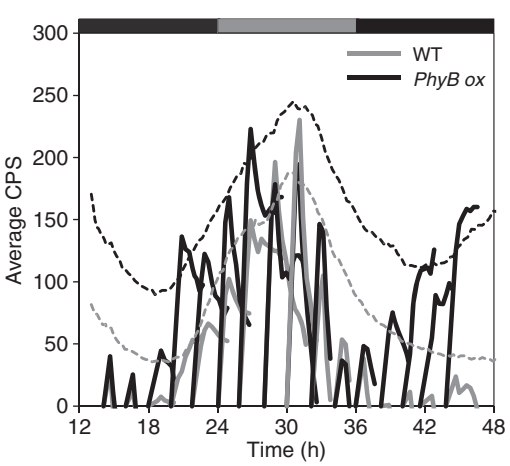

Figure 1. *CAB:LUC* induction is still gated in phyB-overexpressing plants.
Arabidopsis *CAB:LUC* plants with (phyB ox, black traces) or without (WT, grey traces) a 35S:*PHYB* transgene were entrained to LD (12 h light/12 h dark) for 6 days. At ZT12 on day 6, plants were transferred to DD (constant dark). Replicate samples were then exposed to a 20-min red light pulse every 2 h. Luminescence was measured both before and after the light treatment. The mean resulting induction of *CAB:LUC* luminescence (rising traces) was calculated by subtraction of the basal luminescence level of individual seedlings ($n = 24$) before the light treatment. Dashed lines show the luminescence profiles of dark control samples. Light and dark bars represent subjective day and subjective night, respectively. Data are representative of three independent experiments.

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high induction of *CAB:LUC* was still suppressed in the early part of the subjective night (at 14–16 and 34–36 h; Figure 1). Light inducibility was not strictly related to basal expression in the dark, consistent with previous results (Millar and Kay, 1996). The rise in inducibility was slightly advanced compared with the rise in basal level in the wild type (WT), and this tendency was enhanced (at 18 h and 38–40 h) in *35S:PHYB* (Figure 1). This indicates that the clock interacts with phyB signalling at the post-transcriptional level to effect the rhythmic gating of *CAB:LUC* induction. The complexity of the circadian system was not much reduced by constitutive expression of the photoreceptor.

The circadian system in etiolated seedlings

In order to study the clock under a simplified light input regime, we characterised the circadian system in dark-grown but temperature-entrained seedlings. Seedlings were exposed to warm–cold cycles (WC) of 12 h 24°C:12 h 18°C, then transferred to a constant 22°C in constant darkness. Time 0 h refers to the cold–warm transition at the start of the constant conditions. In contrast to Thines and Harmon (2010), we were unable to monitor rhythmic *CAB:LUC* activity reliably because the signal was at the limit of detection (data not shown). This confirms that light signalling pathways were not activated by the temperature entrainment in our studies. We monitored robust rhythms of *COLD AND CIRCADIAN REGULATED 2* (*CCR2*)*:LUC* and *CCA1:LUC* activity. These rhythms had a lower amplitude than in light-grown samples (cf. Doyle *et al.*, 2002; Hall *et al.*, 2003) and their circadian periods were longer than 24 h (Figure S1a in Supporting Information). *CCR2* mRNA accumulation showed very similar regulation (Figure S1b). The expression of both markers was very weakly rhythmic or arrhythmic in *cca1; lhy* double mutant seedlings (Figure S1a), with no evidence of the short-period rhythms detected in light-grown double mutants (Locke *et al.*, 2005). These results confirmed that temperature entrainment allows etiolated Arabidopsis seedlings to maintain a circadian clock.

In order to characterise the targets of circadian regulation in etiolated seedlings, we tested the rhythms of RNA transcript accumulation using high-density oligonucleotide arrays, from 24 to 48 h after transfer to constant 22°C. Seedlings were harvested at five timepoints (6-h interval) in three independent biological replicates. In etiolated seedlings, 35% of transcripts were scored as present at all time points and 38 transcripts were scored as rhythmic, representing only Approximately 0.5% of the genes assayed or 1.6% of the transcripts scored as present (Figure S2, Table S1). Figure 2(a–c) show expression profiles for selected transcripts. Figure 2(d) shows confirmation of selected expression patterns using quantitative (q)RT-PCR. Data were compared with results for light-grown plants investigated in an equivalent technical platform, which scored 453 rhythmic genes (6% of genes on the array) (Harmer *et al.*, 2000) and also cross-referenced to a study of etiolated seedlings by Michael *et al.* (2008; see Figure S3). Several genes involved in light signalling (*PHYA*, *PHYB*, *SPA1*, *CRY1*, *CRY2*, *NPH1*, *RPT2*) were rhythmic in light-grown plants (Harmer *et al.*, 2000) but not in etiolated seedlings. Although none was rhythmically regulated, these genes were all expressed detectably in etiolated seedlings (Figures 2c and S4). Twenty-eight rhythmic transcripts detected in etiolated seedlings (74%) were also rhythmic in Harmer *et al.* (2000). Within this subset were many of the genes associated with the clock mechanism, including *LHY*, *CCA1*, *GI* and *ELF4*, as well as previously characterised rhythmic regulators such as *PIF4*, *CCR2* and its homologue *CCR1* (*GRP8*; Figures 2a and S2a,b, Table S1). Thus the clock controls a small set of rhythmic genes in the absence of light signals. This subset is enriched for genes involved in the clock mechanism, but also in responses to abiotic stress (Table S1).

ELF3-dependent regulation of light-induced gene expression

To investigate whether the circadian clock also gates light-induced gene expression in etiolated seedlings despite the arrhythmic expression of light signalling genes, WC-entrained etiolated seedlings were treated with either red (R) or FR light at either 4 or 16 h. Treatment with either R or FR light at 4 h induced a large acute response of *CAB:LUC* expression that peaked about 2 h after the light pulse. The same light treatment at 16 h induced a fourfold smaller response (Figure 3a,b). The amplitude of the subsequent circadian peak is threefold greater and the peak phase is delayed by 2–3 h in seedlings treated at 16 h in comparison with those treated at 4 h. Similar gating was observed on the next subjective day, comparing treatments at 28 and 40 h (data not shown).

Circadian gating is retained across a wide range of absolute *CAB2* induction levels. Treatment with 200-fold lower R fluence produced ninefold lower peak *CAB* expression compared with Figure 4(a) but still revealed a threefold greater induction after treatment at 4 h than at 16 h (data not shown). The *eid1* mutation sensitises the high-irradiance responses by up to 1000-fold (Buche *et al.*, 2000), with a smaller effect on VLFR such as the induction of *CAB* RNA (Zhou *et al.*, 2002). The level of basal and induced *CAB2* expression was increased significantly (three- to sixfold; Figure 3c,d) in *eid1* compared with WT but circadian gating was at least as effective as in WT (sevenfold between the R treatment at 4 and 16 h in WT in this case, 14-fold in *eid1*).

The *elf3* mutation allows strong induction of *CAB* expression by R light at all circadian phases in light-grown plants (McWatters *et al.*, 2000). We tested whether *ELF3* plays a similar role in *CAB* induction by FR. In the *elf3* mutant, FR light pulses at 4 and 16 h resulted in an identical induction of *CAB:LUC*, with a maximal level of activity about threefold

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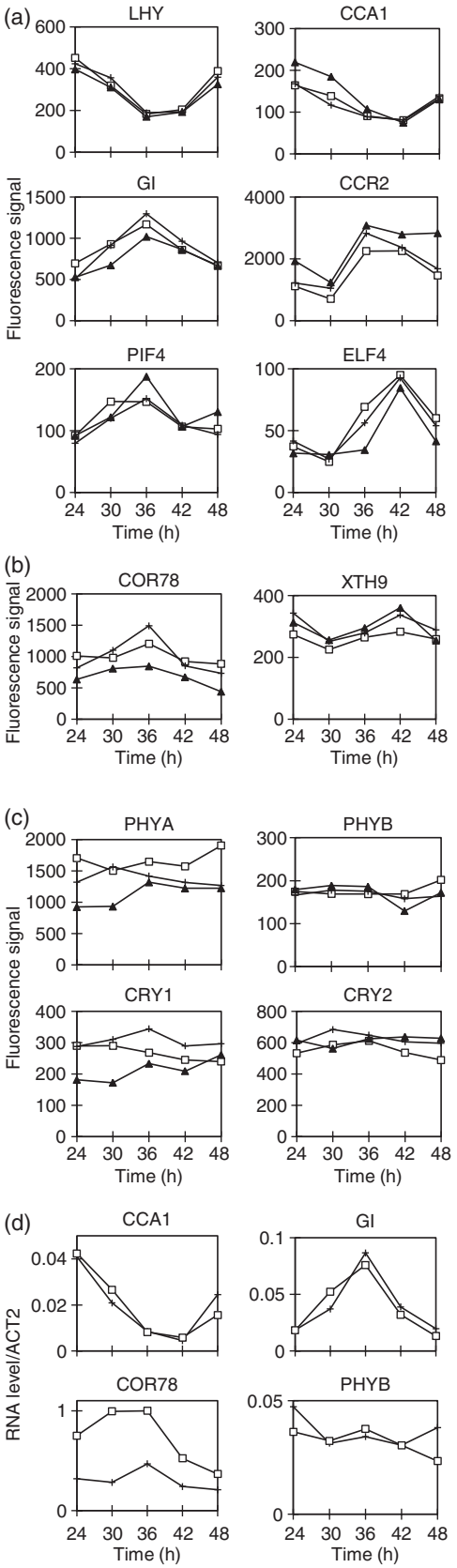

Figure 2. Circadian regulation of gene expression in etiolated seedlings.
The RNA levels were tested using oligonucleotide arrays, from samples of Col-0 seedlings that were entrained for 3 days in warm–cold (WC) cycles, 12 h 24°C:12 h 18°C, and transferred to a constant 22°C at the predicted cold–warm transition (time 0 h).
Analysis of transcripts from selected clock genes (a), rhythmic genes that were not rhythmic in Harmer *et al.* (2000) (b), non-rhythmic photoreceptor genes that were rhythmic in Harmer *et al.* (2000) (c). Data show the fluorescence signal strength for each probe set after microarray analysis (see Supporting Information), in three biological replicates (experiment 1, filled triangles; 2, crosses; 3, open squares).
(d) Quantitative RT-PCR analysis of selected transcripts in experiments 2 and 3 (symbols as above). Data show the abundance of each transcript relative to the *ACT2* control.

greater than in WT seedlings treated at 4 h (Figure 4a). Far-red treatment at 4 h induced *CCA1:LUC* expression in WT plants to peak levels about twofold higher than treatment at 16 h (Figure 4b). The level of maximal induction of *CCA1* was about sixfold lower in *elf3* mutants than in WT treated at 4 h, and light treatments at 4 and 16 h induced identical expression levels in *elf3*. These results show that *ELF3* affects both the level and rhythmic regulation of the response to FR in *CAB:LUC* and *CCA1:LUC* expression.

CCR2 and *CAB2* expression has been reported to be rhythmic in constant darkness (DD) for light-grown *elf3* seedlings (Hicks *et al.*, 1996; Covington *et al.*, 2001), whereas a recent result suggests that the *elf3* mutation led to arrhythmia of *TOC1* expression in WC-entrained etiolated seedlings transferred to constant conditions (Thines and Harmon, 2010). The levels of *CAB:LUC* and *CCA1:LUC* expression before light induction were higher at 4 h than at 16 h in our WC-entrained etiolated *elf3* seedlings transferred to constant conditions, as expected if the plants were rhythmic in this first cycle (Figure 4a). However, this effect of the *elf3* mutation on the response to FR could formally be ascribed to a defect in gating, to a defect in rhythmicity or even to a direct effect on target gene induction that bypasses circadian control.

Thus a rhythmic subset of the clock functioned in darkness, with similar properties to the clock in light-grown seedlings. However, this experimental system used temperature entrainment, potentially as complex as the light inputs. For an alternative, simplified system, we therefore investigated a simple light-entrained clock system with a single input photoreceptor.

Circadian regulation under FR light

Plants grown under FR/D cycles develop similarly to light-grown plants, in contrast to dark-grown seedlings, despite lacking chlorophyll production and photosynthesis. Light input to the clock system was potentially transduced by only the phyA photoreceptor. We therefore tested rhythms of RNA transcript accumulation using high-density oligonucleotide arrays, in seedlings that were entrained under 12 h FR/12 h D cycles for 4 days, from 2 to 50 h after transfer to

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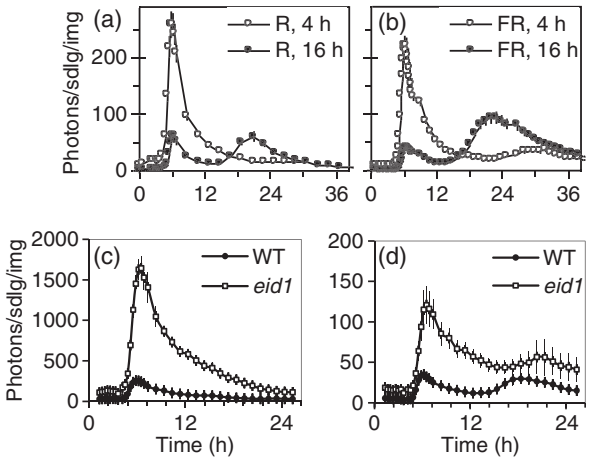

Figure 3. *CAB* induction by red (R) or far-red (FR) light is rhythmically gated in the wild type (WT) and with increased phyA signalling.
Seedlings expressing the *CAB:LUC* reporter gene were entrained and transferred to constant conditions as described in Figure 2, then treated with (a) 10 min R or (b) 10 min FR light at either 4 h (open symbols) or 16 h (filled symbols). Responses to R treatments as above, in WT (filled symbols) or *eid1-3* (*eid1*, open symbols) seedlings at (c) 4 h and (d) 16 h. Data are plotted so as to superimpose the light pulses; the time axis labels refer to the 4-h treatment, 12 h should be added to the time axis for the 16-h treatment. Error bars are 1 SEM. Data are representative of multiple transgenic lines, and of three independent experiments.

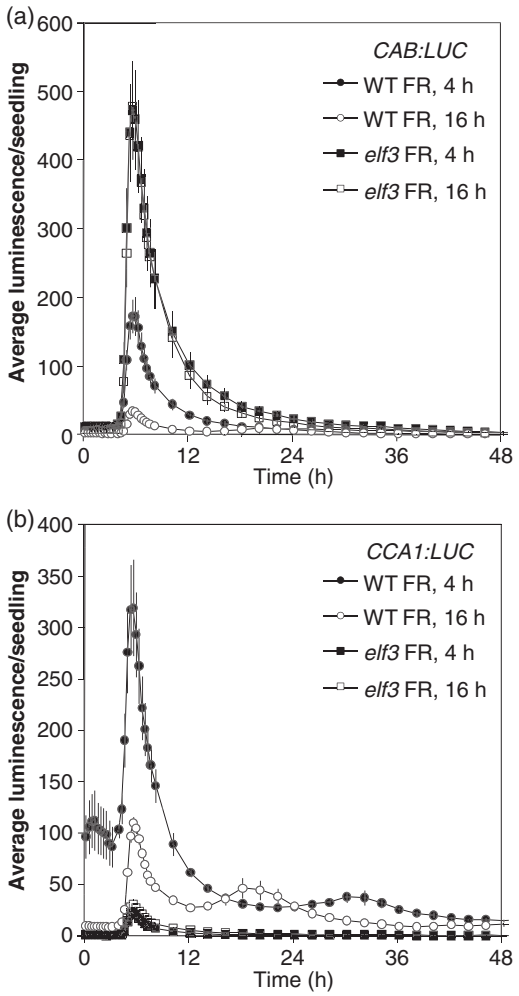

Figure 4. *ELF3* is required for circadian-modulated light induction in etiolated seedlings.
Seedlings expressing (a) the *CAB:LUC* or (b) the *CCA1:LUC* reporter gene in the wild type (WT) or *elf3-4* mutant background were entrained and transferred to constant conditions as described in Figure 2, then treated with 10-min far-red (FR) light at either 4 h (filled symbols) or 16 h (open symbols). Data are representative of three independent experiments.

continuous FR (cFR) light (13 timepoints, 4-h intervals). Many transcripts showed rhythmic expression, but the rhythmic amplitude for all genes was continuously reduced over the time course, which prevented analysis by the approaches used previously (Edwards *et al.*, 2006; Michael *et al.*, 2008).

To illustrate this regulation, we focus on rhythmic genes that displayed an expression peak in the morning (ZT0–6, morning genes) or in the evening (ZT12–18, evening genes) under white light conditions (COSOPT analysis; Edwards *et al.*, 2006; Michael *et al.*, 2008; Table S2). As expected, morning genes displayed a high peak of expression in the first hours of light (Figure 5a) and evening genes were highly expressed around ZT12 (Figure 5b), suggesting that a proportion of the genes scored as rhythmic under white-light-maintained oscillations in FR/D cycles. However, the oscillations in transcript abundance were rapidly damped in cFR (Figure 5). Expression for morning and evening genes stabilised at an average level as early as ZT12 for evening genes (Figure 5b). These results indicate a dramatic response of the clock and clock-related genes to cFR conditions, which we sought to characterise using reporter gene fusions. Figure 6 shows the normalised activity of clock reporter genes monitored under the last entraining FR/D cycle, followed by 96 h of cFR, then 48 h of continuous darkness. As is observed under white light, *CCA1:LUC* displayed an expression peak in the early morning under the FR/D and R/D cycles. *TOC1:LUC*, *GI:LUC* and *CCR2:LUC* expression peaked in the evening (ZT10–12).

All four genes showed disrupted expression from 12 h after transfer to cFR (Figure 6; time 36 h). Consistently with the microarray data for some morning genes, oscillations are damped for *CCA1:LUC* and expression is downregulated, whereas high-amplitude oscillations at higher expression levels were maintained under continuous red (cR) (Figure 6a). For the evening genes, *TOC1:LUC*, *GI:LUC* and *CCR2:LUC*, oscillation amplitude was dramatically decreased and expression was maintained at a high level throughout the cFR treatment compared with cR (Figure 6b–d). After the seedlings were transferred from cFR to continuous dark, all markers dramatically changed expression levels, supporting the idea that the clock perceives the difference between cFR and darkness. For a comparison, FR/D-entrained plants were released into DD for 48 h

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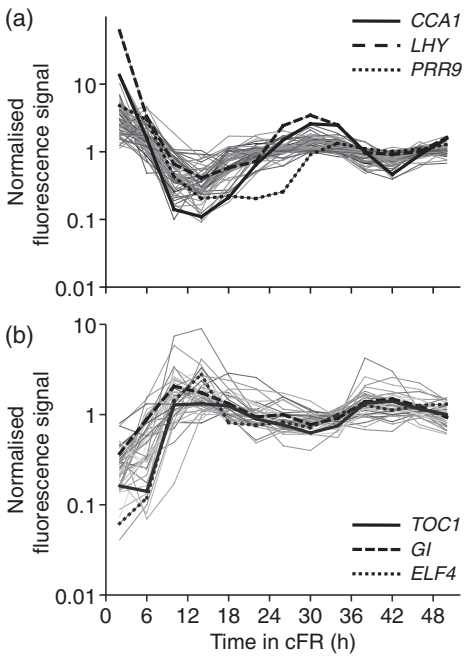


Figure 5. Rhythmic transcripts show unusual regulation in continuous far-red (cFR) light.
The RNA levels were tested using oligonucleotide arrays, from samples of Col-0 seedlings that were entrained under far-red/dark (FR/D) cycles for 5 days and transferred to constant FR light. Rhythmic genes were selected for a fourfold change in expression: (a) morning genes that peak at ZT0–6 and (b) evening genes that peak at ZT12–18. Fifty-five morning genes and 42 evening genes were scored with a fourfold change. Data show a normalised fluorescence signal strength for each probe set after GC – robust multi-array (GC-RMA) analysis.

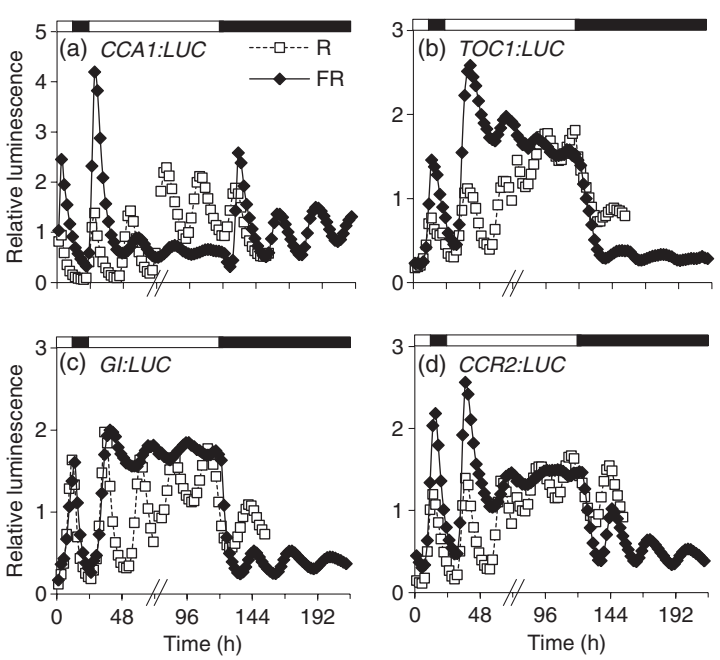
(Figure S5). The activities of the clock gene promoter:LUC remained rhythmic, with low expression levels of *TOC1* and higher levels of *CCA1* and *CCR2*, consistent with the previous experiment. Subsequent transfer to cFR revealed the same responses as in Figure 6, namely a striking suppression of rhythmic amplitude, with low expression levels of *CCA1* and high levels of *GI*, *CCR2* and *TOC1* markers. Additionally, results with lower fluence rates of FR light suggested that the effect of cFR on clock genes was independent of fluence (data not shown).

Functional analysis of rhythmicity in clock mutants under FR light

To investigate whether the clock in cFR is functionally similar to the clock in white light, we monitored *CCA1:LUC* or *CAB:LUC* activity in five clock mutants (Figure 7). Oscillations were maintained in WT and mutants under FR/D cycles but both markers showed disruptions in circadian oscillations in *prr9-1*, *gi*, *elf4-1*, *toc1-1* and *cca1;lhy* mutants in cFR compared with WT. The *CCA1:LUC* period was lengthened in *prr9-1* in cFR (Figure 7a, Table S3) whereas *CCA1:LUC* and *CAB:LUC* were arrhythmic in *gi*, *toc1-1* and *cca1;lhy* mutants, respectively (Figure 7b,d,e, Table S3).

The *elf4* mutation was shown to suppress *CCA1* oscillations and expression level under cR and continuous white light conditions (Doyle *et al.*, 2002; Kikis *et al.*, 2005; McWatters *et al.*, 2007). Consistently with LD-grown (12-h light:12 h dark) seedlings, oscillations for *CCA1:LUC* were maintained in *elf4* under FR/D cycles but were characterised by a lower amplitude than in WT (three- to sixfold lower, Figure 7c, data

Figure 6. Clock gene expression is dramatically affected in continuous far-red (cFR) light.
Seedlings expressing the (a) *CCA1:LUC*, (b) *TOC1:LUC*, (c) *GI:LUC* and (d) *CCR2:LUC* reporter genes were entrained under FR or red/dark (R/D) cycles for 5 days, then imaged under one FR or R/D cycle, transferred to constant R (cR) or cFR light then to continuous dark. Data for FR treatment (closed diamonds) are compared with similar R treatment (open squares). However, for the R experiment, seedlings received 6 days of cR light before being transferred to continuous dark. In order to compare with FR-treated seedlings, the third and fourth days were not included in the graph. Break lines indicate the lapse introduced that correspond to the third and fourth days.



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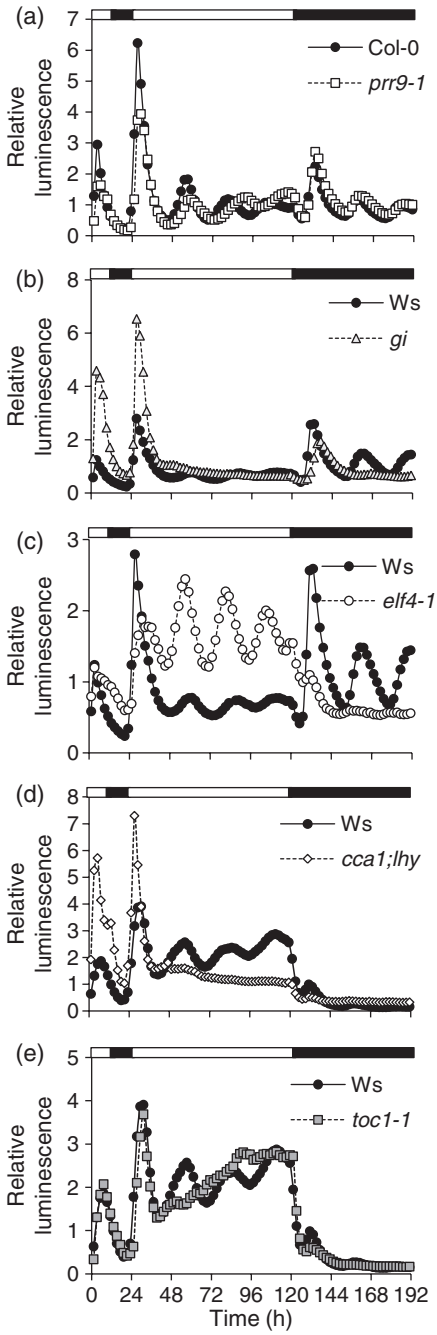

Figure 7. Clock mutants under far-red (FR) treatment.
Seedlings of *prr9-1* (a), *gi-11* (b), *elf4-1* (c) *cca1-11;lhy-21* (d) and *toc1-1* (e), or their cognate wild types (Col-0, Ws), expressing the *CCA1:LUC* (a–c) and the *CAB:LUC* (d, e) reporter gene, were entrained and transferred to constant conditions and tested for luminescence, as described in Figure 6.

not shown). However, *CCA1:LUC* expression level in *elf4* was increased in cFR compared with FR/D cycles and fell in a subsequent dark interval, whereas the level fell in the WT in cFR and rose again in darkness. Most strikingly, oscillation amplitude under cFR tended to be higher in *elf4* mutants compared with WT (Figure 7c), whereas *elf4* is essentially arrhythmic in constant white light. In addition, while both *CCA1:LUC* and *CAB:LUC* markers in Ws and Col-O backgrounds were characterised by a longer period in cFR than in FR/D cycles, oscillations were robust (relative amplitude error (RAE) < 0.25) at a period close to 24 h in *elf4-1* mutant in cFR (Table S3).

As is observed in white light for *CCR2:LUC* in the *cca1;lhy* double mutant (Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002; Locke *et al.*, 2005), oscillations for *CAB:LUC* were maintained in FR/D cycles but damped almost immediately in cFR in *cca1;lhy* double mutant (Figure 7d).

These results suggested that morning loop components, *CCA1/LHY-PRR9/7*, might be functioning similarly in cFR as in continuous white light. In contrast, altered *CCA1:LUC* rhythms in *gi*, *toc1* and *elf4* mutants suggested that evening loop components contribute to altered functions or more specifically to the cFR signalling pathway to the clock.

phyA largely controls the clock under FR conditions

In order to further investigate the role of phyA in FR light input to the clock, we analysed the response of the clock genes to FR/D cycles and cFR in the *phyA-211* mutant. Seedlings were treated as described previously and harvested at 2-h intervals under one FR/D cycle, followed by 72 h of cFR and 48 h of DD in two independent biological replicates. Both Col-0 seedlings and the *phyA-211* mutants displayed an etiolated morphology in these conditions, which used a filtered, low-fluence-rate FR treatment (peak emission at 760 nm, see Experimental Procedures) designed to test phyA specificity (Figure S6). We tested whether the unusual light conditions caused oxidative stress that might affect our results, but the stress-responsive At*TRXh5* transcript was not induced (Figure S7) (Laloi *et al.*, 2004).

Figure 8 shows qRT-PCR analyses of transcript levels for six clock genes in *phyA-211* and Col-0. *CCA1* and *LHY* expression in Col-0 displayed high-amplitude oscillations in FR/D cycles that were damped under cFR and restored after transfer to DD, confirming the previous observations that morning genes are downregulated in cFR conditions. Strikingly, in *phyA-211*, *CCA1* and *LHY* expression was dramatically reduced but low-amplitude oscillations were still detectable, though the oscillation amplitudes were similar under FR/D cycles, cFR and subsequent DD conditions. These results indicate that phyA is important for the suppressive effect of cFR on morning gene expression in the WT.

Oscillations were maintained for *PRR9* under FR/D cycles in Col-0 but its expression was not downregulated in cFR. The amplitude of oscillations in *PRR9* expression was decreased in cFR but the average level of transcript abundance rose above the mean in FR/D (Figure 8). Mutation of *PHYA* greatly decreased *PRR9* expression and inhibited oscillations almost completely in cFR.

Microarray and luciferase results suggested that evening-expressed genes were rhythmic in WT under FR/D cycles and

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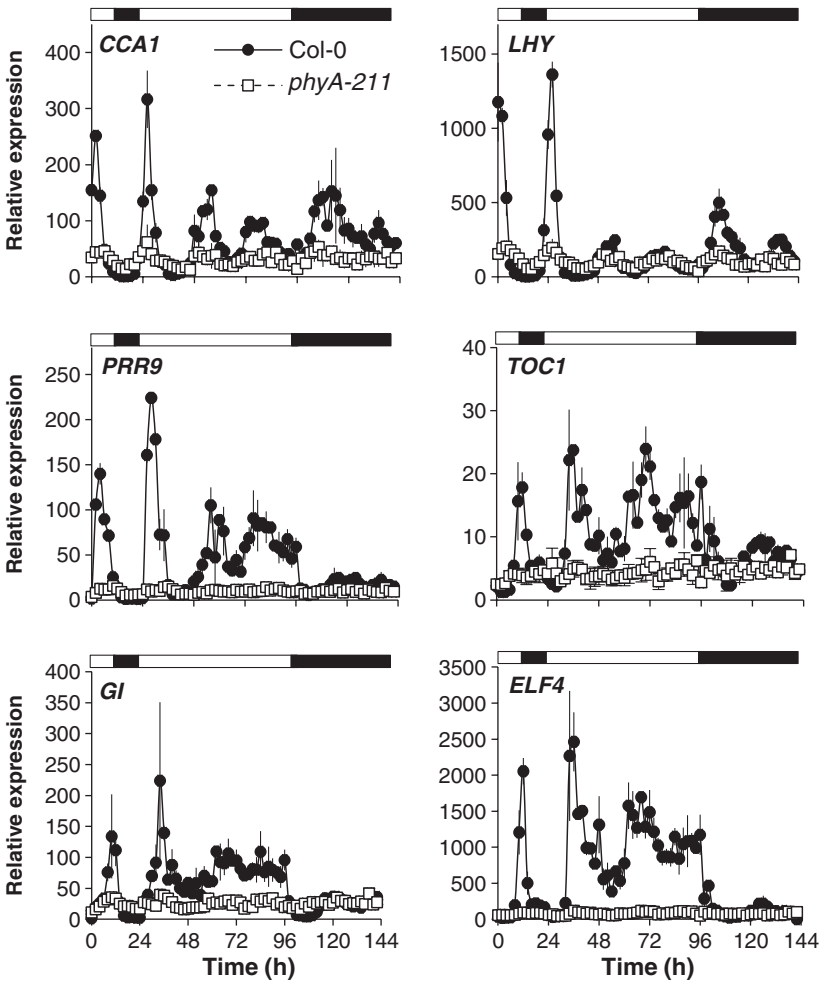

Figure 8. Clock gene expression is dramatically altered in *phyA* mutants under far-red (FR) light. Transcript levels from selected genes were tested using quantitative RT-PCR, from samples of Col-0 and *phyA-211* seedlings that were entrained for 5 days in far red/dark (FR/D) cycles, and transferred to continuous FR then DD. Data show the abundance of each transcript relative to the *ACT2* control.

upregulated under cFR treatment. These observations were confirmed by qRT-PCR for *TOC1*, *GI* and *ELF4* (Figure 8), with detectable but low-amplitude oscillations under continuous conditions. *TOC1* and *GI* expression in *phyA-211* was downregulated just above the trough level in WT (Figure 8), with little or no detectable rhythmicity, suggesting that their expression is normally activated largely by phyA. *ELF4* expression was almost abolished in the *phyA* mutant, showing that *PHYA* is essential for *ELF4* expression under FR light conditions.

Overall, these data provide evidence that *PHYA* is a primary component of FR light input in FR/D cycles but might not be the only active photoreceptor, at least for *CCA1* and *LHY*. *PHYA* is, however, essential for the unusual clock gene regulation under cFR conditions.

DISCUSSION

Circadian clock mechanisms include gene regulation by multiple, interlocking feedback loops, which can increase the flexibility of possible regulatory changes over evolutionary time (Rand *et al.*, 2004; Edwards *et al.*, 2010) and the robustness to environmental noise (Troein *et al.*, 2009). Multiple feedback loops have been implicated in the circadian system of light-grown Arabidopsis seedlings (Locke *et al.*, 2006; Pokhilko *et al.*, 2010). Multiple photoreceptors contribute light input signals, adding further complexity to the clock network. In order to simplify the light inputs, we characterised the circadian system in dark- and FR-grown seedlings. In dark-grown seedlings, the clock mechanism regulates a much smaller set of rhythmic target genes than in light-grown plants. A simple FR light input system revealed unexpected interaction between FR light input and clock components. Overall, in both systems, the circadian clock mechanism shared central components with the clock in 'lab-standard' light-grown plants, but the influence of the light inputs was strong enough to profoundly alter circadian function.

A simpler circadian system retains key features of the clock in light-grown tissues

Warm–cold cycles of modest amplitude (6°C) entrained the circadian clock reproducibly in etiolated Arabidopsis seedlings, whereas arrhythmic expression of clock genes has

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previously been reported for non-entrained etiolated Arabidopsis (Kikis *et al.*, 2005). The clock in etiolated seedlings shares some components with light-grown plants, because the *ztl* (Kevei *et al.*, 2006) and *elf3* mutants (this work; Thines and Harmon, 2010) clearly affect etiolated plants. Single mutants in *PRR5* or *PRR9* had no detectable effect in etiolated seedlings but showed mild clock phenotypes in light-grown plants (Eriksson *et al.*, 2003). The *lhy*; *cca1* double mutant had a stronger effect in etiolated plants, abolishing rhythms rather than leaving the damped, 18-h rhythms typical of light-grown material (Alabadi *et al.*, 2002; Mizoguchi *et al.*, 2002; Locke *et al.*, 2005), though *lhy* and *cca1* single null mutants did not have this severe effect (data not shown). The clock in dark-grown root material was also more dependent on *LHY* and *CCA1* functions (James *et al.*, 2008). Thus the circadian system in dark-grown plants lacks the circuit that supports short-period rhythms in light-grown *lhy;cca1* seedlings, possibly because this circuit involves light-activated components, such as *PRR9*.

Transcriptome analysis showed that the circadian clock in etiolated seedlings regulated only a small subset of the rhythmic genes identified in light-grown plants: 6% of the rhythmic transcripts of Harmer *et al.* (2000), who assayed the same set of transcripts in light-grown plants. These results support observations of Michael *et al.* (2008), who scored 6% of transcripts as circadian-regulated in etiolated seedlings entrained to 10°C hot–cold cycles and transferred to constant temperature, compared with 19–31% in light-dark-grown seedlings in constant light (Michael *et al.*, 2008).

Transcripts of several genes involved in light signalling accumulate to significant levels but are non-rhythmic as confirmed by qRT-PCR, consistent with reporter gene data for *PHYA* and *PHYB* (Kolar *et al.*, 1998). The circadian regulation of these genes in light-grown plants is therefore dependent upon active light signalling, consistent with the light-dependence of some central clock functions discussed above. Circadian gating of plant responses to light is one overt consequence of the rhythmic control of light-signalling components (Millar and Kay, 1996). Circadian gating was unlikely to be mediated directly by rhythmic photoreceptor gene expression, because neither *PHYA* nor *PHYB* transcript levels were rhythmically regulated in etiolated seedlings, in contrast to light-grown plants (Hall *et al.*, 2001; Toth *et al.*, 2001). This is also consistent with maintained gating in plants that constitutively expressed *PHYB*, suggesting that gating regulates phyB signalling at a post-transcriptional level. The circadian clock in etiolated seedlings gated the acute induction of *CAB2* gene expression, allowing greater induction by R and FR in the subjective day than in the subjective night. The subsequent circadian-regulated peak of gene expression was reciprocally regulated (higher expression after light treatment during the night), confirming that this aspect of gene expression is not simply coupled to the acute response to light (Anderson *et al.*, 1997).

Overall, temperature entrainment allows the circadian clock to function in darkness with similar general properties to the clock in light-grown seedlings, including a remarkable capacity to modulate light responses. However, its sensitivity to mutation and its target genes are significantly different from the light-entrained clock, and the mechanisms of temperature input remain obscure and are potentially as complex as the light inputs. An experimental clock system entrained by a limited, defined light input might better represent the clock in standard, white-light-grown plants than the etiolated clock.

The circadian clock under FR light input

The RNA transcript accumulation, luciferase and quantitative RT-PCR assays from seedlings that were transferred to cFR light after being entrained under FR/D cycles showed high-amplitude oscillations for the main clock genes, suggesting that the circadian system under FR/D cycles was similar to that in plants grown in white light. Under cFR light, however, the amplitude of oscillation for both morning and evening genes was dramatically reduced immediately after one cycle of cFR, whereas oscillations for rhythmic genes were maintained for several cycles under constant R or white light.

The morning genes *CCA1* and *LHY* were downregulated, whereas *PRR9* was, if anything, upregulated. A simple interpretation suggests that the *CCA1* and *LHY* downregulation observed in cFR could lead to a lack of *PRR9* activation (Farré *et al.*, 2005). Direct light regulation of *PRR9* (Ito *et al.*, 2003) is likely to be more significant, as *PRR9* was upregulated in cFR. *PRR9* expression was inhibited in the *phyA* mutant, consistent with strong activation by FR light through phyA. Removal of the morning loop in the double mutant *lhy;cca1* led to complete arrhythmia for *CAB:LUC* in cFR whereas oscillations were maintained in FR/D. This suggests that the morning loop is essential for oscillations under cFR conditions, as in dark-grown seedlings.

In cFR, evening clock genes were expressed at high levels, with oscillations of low amplitude. *GI* and *ELF4* expression is known to be activated by light (Fowler *et al.*, 1999; Kikis *et al.*, 2005; Locke *et al.*, 2005) hence, similarly to *PRR9*, upregulation of *GI* and *ELF4* expression under cFR conditions could be the direct consequence of a strong FR light activation that required phyA. *TOC1* expression, in contrast, is not acutely light-responsive (Makino *et al.*, 2001) but shows the same high, phyA-dependent expression as the light-activated genes. Both *toc1* and *gi* mutants were arrhythmic in cFR, in contrast to their maintained rhythms in constant white light, showing that evening functions are still required for rhythmicity.

To learn from the system's behaviour in cFR will require an understanding of higher-order gene interactions. *TOC1* is negatively regulated by *LHY* and *CCA1* (Matsushika *et al.*, 2000; Strayer *et al.*, 2000), so upregulated *TOC1* expression

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alone cannot distinguish between a direct activation by FR, an indirect activation by another component, or a consequence of low inhibition due to the low *CCA1* and *LHY* levels in cFR. Whereas *CCA1* and *LHY* were downregulated, expression of their inhibitor *PRR9* was maintained at a higher level, as were the evening-expressed genes *GI*, *TOC1* and *ELF4* that normally activate *LHY* and *CCA1*. The lack of *CCA1* and *LHY* expression suggests that their potential activators were blocked under cFR conditions. This is consistent with the observation that the *prr9* mutant showed a longer period phenotype in cFR, similar to observations for *prr9* in constant white light (Ito *et al.*, 2003), indicative of strong *PRR9* function in the wild type in cFR. The striking restoration of rhythms under cFR in the *elf4* mutant, with increased *CCA1* expression, suggests that *ELF4* was required for the function of the inhibitors, rather than functioning directly as an activator. The normal activation of *CCA1* by *ELF4* (Doyle *et al.*, 2002; Kikis *et al.*, 2005) would require a double-negative interaction, in this hypothesis, in which *ELF4* inhibited the expression of the *LHY/CCA1*-inhibitors, such as *PRR9*. This would be similar in principle to the inhibitory role proposed for *ELF3* (Thines and Harmon, 2010).

The effect of cFR can be understood as preventing one step in the normal circadian cycle of activator and inhibitor expression, holding the clock at one part of the limit cycle. The fact that such a disrupted system still produces a normal period of oscillation is itself remarkable. The high amplitude of clock gene oscillations under FR/D cycles, compared to very low rhythmic amplitude in cFR, suggests that this critical step must normally occur at night. For example, dark-dependent degradation of the inhibitor(s) of *LHY* and *CCA1* might allow the expression of *LHY* and *CCA1* before dawn. Indeed, PRR proteins do exhibit such dark-dependent degradation (Ito *et al.*, 2007). The WC-entrained etiolated seedlings did not show these very low-amplitude rhythms, with high evening gene expression and low *LHY* and *CCA1*. Therefore active phytochrome signalling was required for the effects observed in cFR.

Our results did not identify any gene that is uniquely required for FR input to the clock, as judged by the strongest criterion that a mutation in such a gene should abolish both entrainment by FR/D cycles and the characteristic damping in cFR. The very strong, phyA-dependent regulation of *ELF4* mRNA makes *ELF4* itself a strong candidate for one point of cFR input to the clock. The *elf4* mutant was still entrained in FR/D cycles, indicating that *ELF4* is not uniquely required for entrainment by FR. To our knowledge, however, no other light condition was found to have such a strong effect on the clock genes. The cFR conditions characterised here not only illustrate how profoundly the light input pathways affect the plant circadian clock, but also provide a strong experimental manipulation for understanding at least one critical step in the clock mechanism.

EXPERIMENTAL PROCEDURES

Plant material

CAB:LUC lines expressing 35S::*PHYB* were previously described (Hall *et al.*, 2002). The *prr9-1* mutant is in the Columbia-0 background (Eriksson *et al.*, 2003); *phyA-211* is also in Columbia (Reed *et al.*, 1994). All other transgenic Arabidopsis lines were in the Wassilewskija accession. The *CAB2:LUC+*, *CCR2:LUC+*, *CCA1:LUC+*, *TOC1:LUC+* and *GI:LUC+* lines and their introduction into *elf3-4*, *elf4-1, prr9-1*, *toc1-1* and *gi-11* backgrounds have been described, together with the double mutant *cca1-11; lhy-21* (Doyle *et al.*, 2002; Eriksson *et al.*, 2003; Hall *et al.*, 2003; Gould *et al.*, 2006; Edwards *et al.*, 2010).

Growth conditions

Growth conditions for samples used in leaf movement, luciferase and RNA studies were essentially as described, on solid Murashige–Skoog agar medium containing 3% sucrose (Edwards *et al.*, 2005, 2010). Variations for specific experiments are described in the Supporting Information.

Measurement of leaf movement and gating assays

Individual period estimates were produced from leaf movement data as described (Edwards *et al.*, 2005). Gating assays were as previously described (McWatters *et al.*, 2000).

Microarray data

Growth conditions and data analysis are described in the Supporting Information.

Luminescence image analysis

Seedlings were sprayed with 5 mM luciferin solution in 0.01% Triton-X100 in complete darkness approximately 24 h before the start of the imaging assay. Seedlings were arranged in clusters of 20–30 within transparent plastic collars that prevent lodging during the experiment. Foil baffles were placed between different transgenic lines to stop luminescence cross-talk. Luminescence images were captured and analysed essentially as described (Edwards *et al.*, 2005, 2010). Mean period estimates for each genotype were based on six to eight individual plants as described (Edwards *et al.*, 2005, 2010).

Quantitative RT-PCR

Approximately 75–100 seedlings were harvested in RNAlater (Sigma, http://www.sigmaaldrich.com/) and total RNA was extracted using a Plant RNeasy kit (Qiagen, http://www.qiagen.com/) with on-column DNase digestion. The cDNA samples for real-time PCR applications were reverse transcribed from 1 μg of RNA using the SuperScript® VILO® cDNA Synthesis kit (Invitrogen, http://www.invitrogen.com/), and the cDNA product was diluted 1:10 in RNase-free water. The qPCR was set up with a liquid handling robot (TECAN freedom Evo, http://www.tecan.com/) and cDNAs for each sample were quantified in triplicate using the LightCycler® 480 Real-Time PCR System (Roche, http//http://www.roche.com/). Primers used are described in the Supporting Information.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Etiolated seedlings maintain temperature-entrainable circadian rhythms.

Figure S2. Circadian regulation of gene expression in etiolated seedlings.

Figure S3. Expression in etiolated seedlings from Michael *et al.*, 2008 for genes that were found rhythmic in the present study.

Figure S4. Threshold for rhythmic scoring.

Figure S5. Clock genes expression patterns do not result from conditions prior to the transfer to continuous far red (cFR) or the seedlings age.

Figure S6. Phenotype of Col-0 and *phya* seedlings under low-fluence far-red/dark (FR/D) cycles used for the analysis of transcripts from selected clock genes.

Figure S7. Continuous far-red (cFR) light conditions do not increase expression of the oxidative stress gene *TRX5.*

Table S1. Clock-controlled genes in etiolated seedlings.

Table S2. Morning and evening genes subsets selected for far-red/dark (FR/D) grown seedlings transferred to continuous far-red (cFR) light.

Table S3. Period analysis of circadian clock mutants under constant far-red light conditions.

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