

Coordinated transcriptional regulation underlying the circadian clock in *Arabidopsis*

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The circadian clock controls many metabolic, developmental and physiological processes in a time-of-day-specific manner in both plants and animals^{1,2}. The photoreceptors involved in the perception of light and entrainment of the circadian clock have been well characterized in plants³. However, how light signals are transduced from the photoreceptors to the central circadian oscillator, and how the rhythmic expression pattern of a clock gene is generated and maintained by diurnal light signals remain unclear. Here, we show that in *Arabidopsis thaliana*, FHY3, FAR1 and HY5, three positive regulators of the phytochrome A signalling pathway, directly bind to the promoter of *ELF4*, a proposed component of the central oscillator, and activate its expression during the day, whereas the circadian-controlled CCA1 and LHY proteins directly suppress *ELF4* expression periodically at dawn through physical interactions with these transcription-promoting factors. Our findings provide evidence that a set of light- and circadian-regulated transcription factors act directly and coordinately at the *ELF4* promoter to regulate its cyclic expression, and establish a potential molecular link connecting the environmental light–dark cycle to the central oscillator.

Circadian rhythms are synchronized by the day–night cycle so that organisms can correctly anticipate the cyclic changes of the external environment⁴. In plants, the circadian clock plays a key role in regulation of flowering, water use efficiency, coordinating internal and external cues governing rhythmic elongation growth, and responses to hormones and light^{1,5}. The plant circadian clock is composed of multiple positive and negative factors organized in interlocked transcriptional and post-translational feedback loops^{5,6}. In the central clock of *Arabidopsis thaliana*, two morning-phased transcription factors CCA1 (Circadian Clock-Associated 1) and LHY (Late Elongated

Hypocotyl) repress the transcription of the evening-phased gene *TOC1* (*Timing of Cab Expression 1*) by recognizing the evening element (EE) present in the *TOC1* promoter⁷. *TOC1* physically interacts with CHE, a TCP transcription factor that directly binds to the *CCA1* promoter⁸, and promotes *CCA1* expression, thus forming a transcriptional feedback loop. *ELF4* (*Early Flowering 4*), another evening-phased gene⁹, is essential for the expression of *CCA1* and *LHY* and maintenance of the circadian rhythm, whereas expression of *ELF4* is negatively regulated by *CCA1* and *LHY* (refs 10,11). Thus, it has been proposed that *ELF4* represents another component of the central oscillator, and the negative feedback loop formed by *CCA1*–*LHY* and *ELF4* is analogous to the *CCA1*–*LHY* and *TOC1* loop^{10,11}. However, the positive regulators responsible for directly activating the expression of evening-phased genes (*TOC1* and *ELF4*) have remained elusive.

FHY3 (Far-red Elongated Hypocotyl 3) and its paralogue FAR1 (Far-red Impaired Response 1), two transposase-derived transcription factors, are essential for regulating phytochrome A (phyA)-mediated seedling photomorphogenic responses in *Arabidopsis*¹². FHY3 has a role in gating phytochrome signalling to the circadian clock¹³. Quantitative PCR with reverse transcription (qRT–PCR) analysis revealed that the expression levels of the morning-expressed *CCA1* and *LHY* showed a significant reduction in *fhy3* and *far1* mutants after two cycles in the free-running assay under continuous white-light conditions (Fig. 1a,b). The expression level of the evening-expressed gene *ELF4* damped more rapidly, showing a marked reduction after one cycle and was nearly abolished after two cycles in *fhy3* and *far1* mutants (Fig. 1c). Furthermore, expression levels of *Catalase 3* (*CAT3*) and *TOC1*, two other evening-expressed genes, are also markedly affected in *fhy3* mutants and become arrhythmic after one or two cycles in the free-running assay, respectively (Fig. 1d,e). However, a morning-expressed gene, *Chlorophyll a b-binding protein 2* (*CAB2*), is less affected (compared with *CAT3* and *TOC1*) in *fhy3* mutants,

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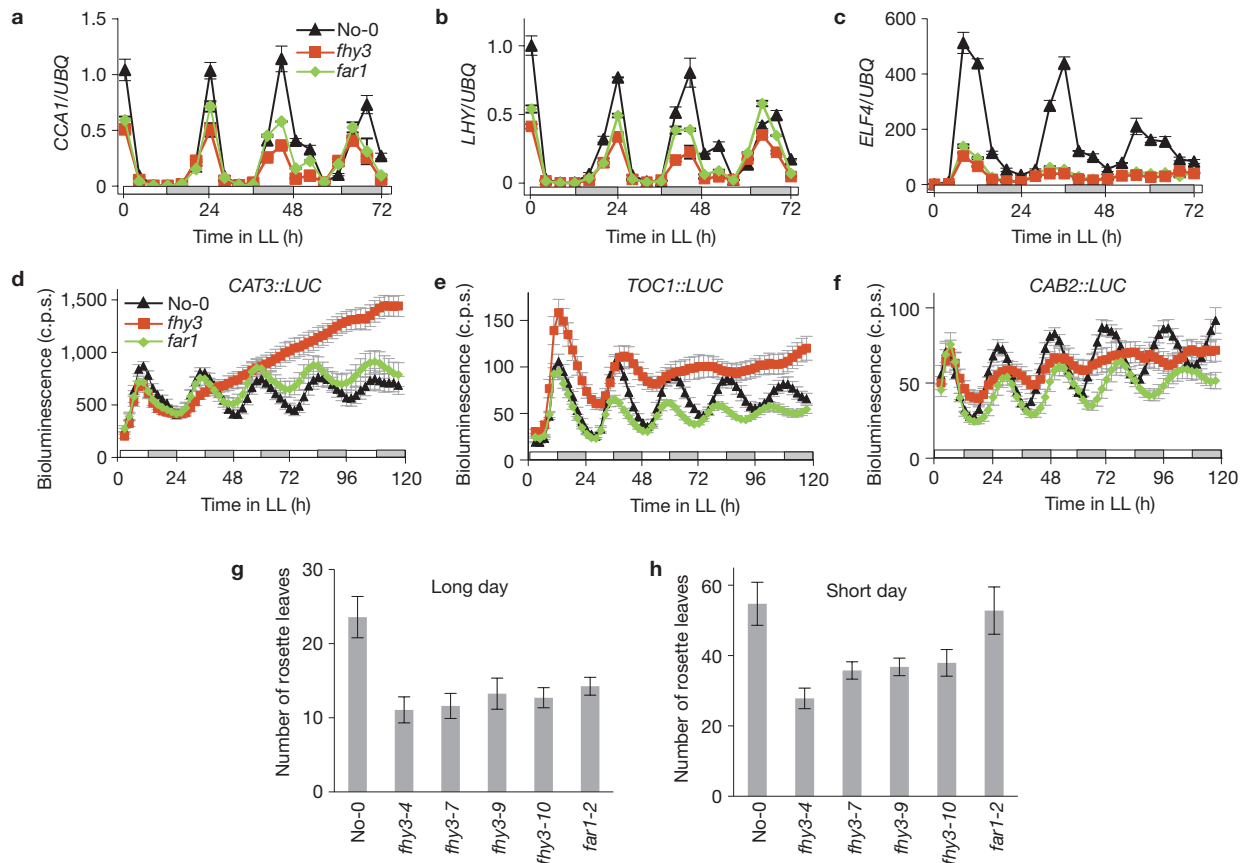


Figure 1 FHY3 and FAR1 are essential for circadian gene expression and flowering-time control. (a–c) qRT-PCR analyses showing that cyclic expression of *CCA1*, *LHY* and *ELF4* was reduced in *fhv3* and *far1* mutants in continuous white-light (LL) conditions (mean \pm s.d., $n = 3$). (d,e) Bioluminescence assays showing that expression of *CAT3::LUC* and *TOC1::LUC* reporters was arrhythmic in the *fhv3* mutants in continuous white-light conditions. (f) Expression of *CAB2::LUC* showed a reduction

in amplitude and lengthening of period. c.p.s., counts per second (mean \pm s.e.m., $n = 18$). The white and grey bars in each panel indicate the subjective day and night, respectively. (g,h) *fhv3* mutants flowered early under both long-day (16 h light/8 h dark) and short-day (8 h light/16 h dark) conditions. The numbers of rosette leaves at the time of the first flower opening are shown (mean \pm s.d. g, $n = 19$ for *fhv3-9*, $n = 30$ for other lines; h, $n = 24$). No-0; Nossen ecotype, wild type.

although it showed a reduction in amplitude and lengthening of period (Fig. 1f). These observations indicate that FHY3 and FAR1 are two essential regulators of central clock gene expression.

In addition to showing defects on circadian gene expression, *fhv3* and *far1* mutants flowered much earlier and possessed fewer rosette leaves than wild-type plants at bolting under both long-day (16 h light/8 h dark) and short-day (8 h light/16 h dark) conditions, although flowering time was less affected in the *far1* mutant under short-day conditions (Fig. 1g,h). Flowering is one of the most important seasonal growth processes that is mediated through coupling of endogenous rhythms and external light signals^{14–16}. Consistent with this, the expression of *CAT3*, *TOC1* and *CAB2* is less affected in the *far1* single mutant (Fig. 1d–f). These observations indicate that FHY3 plays a more predominant role in the circadian clock and flowering time control.

FHY3 and FAR1 can directly bind to the *ELF4* promoter *in vitro*¹², indicating that *ELF4* may be a direct downstream target of FHY3 and FAR1. Promoter analysis revealed that there are two FHY3–FAR1-binding sites (FBS, CACGCGC, nucleotides –192 to –186 and nucleotides –183 to –189), three evening elements (EE, CCA1–LHY-binding sites AAATATCT, nucleotides –72 to –79, nucleotides –306 to –313 and nucleotides –323 to –316) and two ACGT-containing elements (ACEs, nucleotides –158 to –155 and

nucleotides –164 to –161) in the 400-base-pair proximal region of the *ELF4* promoter (Fig. 2a and Supplementary Fig. S1a). HY5 (Long Hypocotyl 5), a bZIP (basic domain leucine zipper)-type transcription factor, can bind to ACE elements in the promoters of light-responsive genes and promote photomorphogenesis¹⁷. Yeast one-hybrid assays and electrophoretic mobility shift assays (EMSAs) showed that mutations in the FBS sites, EE elements or ACEs elements in this region significantly reduced or abolished the DNA-binding activities of FHY3 and FAR1, CCA1 and LHY, or HY5, respectively, indicating that the FBS, EE and ACE elements are critical for specific binding of FHY3 and FAR1, CCA1 and LHY, and HY5 to the *ELF4* promoter *in vitro* (Fig. 2a and Supplementary Fig. S1b).

To further investigate the binding of FHY3, FAR1, CCA1 and HY5 to the *ELF4* promoter, we carried out a chromatin immunoprecipitation quantitative PCR (ChIP–qPCR) assay using wild-type plants and transgenic seedlings expressing $3 \times$ Flag–FHY3– $3 \times$ HA (Supplementary Fig. S2a), *FAR1::FAR1–Myc* (Supplementary Fig. S2b), *HY5::HY5–YFP* (ref. 18) or *CCA1::GFP–CCA1* (ref. 8). This assay revealed a specific enrichment of *ELF4* promoter fragments containing the FBS, EE and ACEs elements, respectively (Fig. 2b and Supplementary Fig. S2c), indicating that FHY3, FAR1, CCA1 and HY5 directly bind to the *ELF4* promoter *in vivo*. In addition,

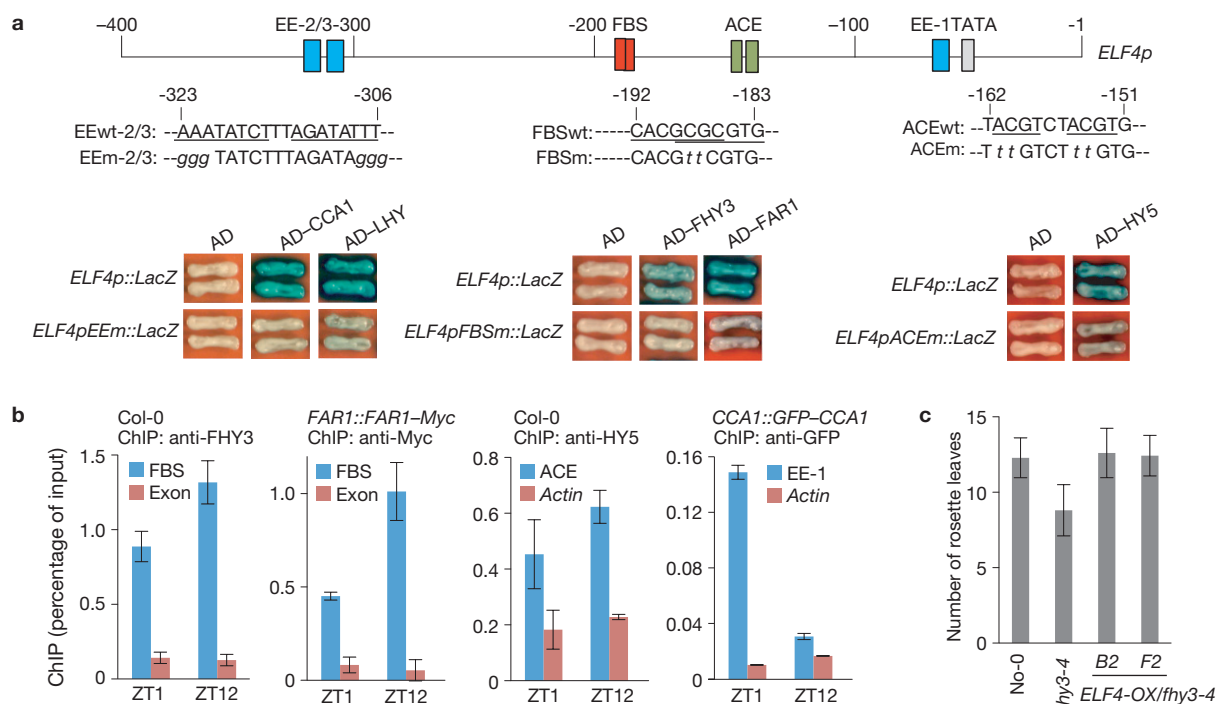


Figure 2 FHY3, FAR1, CCA1, LHY and HY5 directly bind to distinct *cis* elements in the *ELF4* promoter *in vitro* and *in vivo*. (a) Top panel, schematic representation of the position and nucleotide sequence of various *cis*-elements in the *ELF4* promoter. wt, wild-type; m, mutant. Bottom panels, yeast one-hybrid assays showing that CCA1, LHY, FHY3, FAR1 and HY5 activate the *LacZ* reporter genes driven by the full-length wild-type *ELF4* promoter, but not *LacZ* reporter genes driven by the *ELF4* promoter with mutations in the specific *cis* elements. mEE, mutation of all three EE elements (EE-1, EE-2 and EE-3). AD, activation domain.

(b) ChIP-qPCR assays showing that the *ELF4* promoter fragments containing the putative FHY3-, FAR1-, HY5- and CCA1-binding sites are specifically enriched. A carboxy-terminal exon region (Exon) of *ELF4* and *Actin* were used as the negative controls for the ChIP-qPCR experiment (mean \pm s.d., $n = 3$). Col-0; Columbia ecotype, wild type. (c) Overexpression of *ELF4* (*ELF4-OX*) rescued the early-flowering phenotype of the *fhy3* mutant under long-day conditions. B2 and F2 are two independent transgenic lines (mean \pm s.d., $n = 19$ for No-0 and B2; $n = 15$ for *fhy3-4* and F2).

overexpression of *ELF4* in the *fhy3-4* mutant background completely rescued the early-flowering phenotype of the *fhy3* mutant (Fig. 2c), indicating that regulation of flowering time by FHY3 is at least partially mediated through *ELF4*.

To investigate the molecular mechanism of transcriptional regulation of *ELF4* *in vivo*, we generated transgenic plants expressing a *LUC* reporter gene driven by full-length wild-type and various mutated forms of the *ELF4* promoter. Expression of *ELF4::LUC* in the *fhy3 far1* double mutant or *LUC* reporter genes driven by the *ELF4* promoter containing mutations in the FBS elements (*FBSm::LUC*) showed a rapid damping and an arrhythmic expression pattern after one cycle in the free-running assay (Fig. 3a,c), indicating that FHY3 and FAR1 are essential for both the amplitude and rhythmic expression of *ELF4*. On the contrary, expression levels of *ELF4::LUC* in the *hy5 hyh* double-mutant background or a *LUC* reporter gene driven by the *ELF4* promoter containing mutations in the ACE elements (*ACEm::LUC*) both showed much reduced amplitudes, but with normal rhythmic expression patterns (Fig. 3b,c), indicating that HY5 is mainly required for the amplitude of *ELF4* expression, but is not essential for its rhythmicity.

ELF4 expression is negatively regulated by CCA1 and LHY (ref. 10). To verify this, we examined *ELF4* expression level in plants overexpressing CCA1 (CCA1-OX;¹⁹) using qRT-PCR. We observed a significant reduction of *ELF4* expression in CCA1-OX plants (Fig. 3d), thus confirming that CCA1 is a negative regulator of *ELF4* expression.

Next, we carried out a transient expression experiment in *Nicotiana benthamiana* leaves to directly test the role of FHY3, FAR1, HY5, CCA1 and LHY on *ELF4* expression. Expression of a *LUC* reporter gene driven by the *ELF4* promoter was significantly elevated by co-expression of FHY3, FAR1 and HY5, but much reduced by co-expression of CCA1 or LHY (Fig. 3e,f). These results further support the hypothesis that FHY3, FAR1 and HY5 are direct transcriptional activators of *ELF4* expression whereas CCA1 and LHY are direct transcriptional repressors of *ELF4* expression.

Previous studies have reported physical interaction between FHY3 and FAR1 (ref. 12), CCA1 and LHY (refs 20,21) and CCA1 with HY5 (ref. 22). Thus, we next tested whether FHY3 and FAR1 can interact with CCA1, LHY and HY5. Our yeast two-hybrid assay showed that FHY3, but not FAR1, directly interacts with CCA1, LHY and HY5 (Fig. 4a). A co-immunoprecipitation assay using transgenic *Arabidopsis* plants expressing $3 \times$ Flag-CCA1- $3 \times$ HA (Supplementary Fig. S3a) also confirmed *in vivo* interaction between CCA1 and FHY3 proteins (Fig. 4b). Furthermore, a luciferase complementation image (LCI) assay revealed that the amino termini of FHY3 and CCA1, which contain their respective DNA-binding domain, are responsible for mediating their interaction (Fig. 4c). Collectively, these data indicate that these transcription factors may work together to coordinately regulate *ELF4* expression. Consistent with this hypothesis, a gel-filtration chromatography assay further confirmed that FHY3 and LHY are

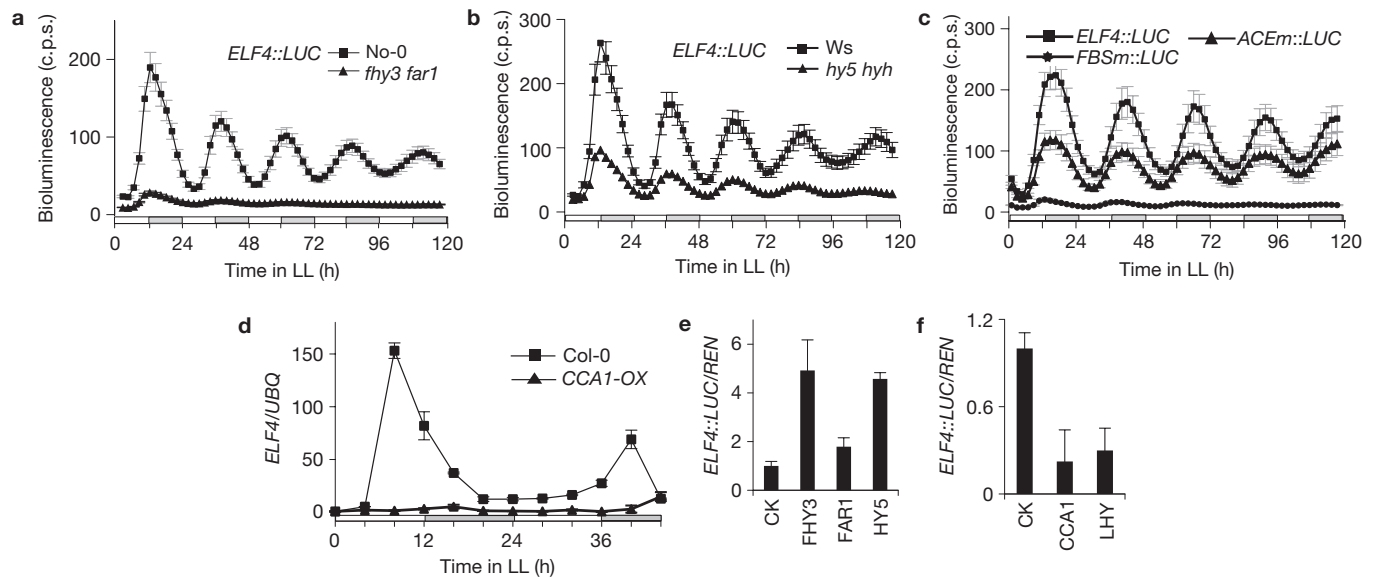


Figure 3 FHY3, FAR1 and HY5 activate *ELF4* expression, whereas CCA1 and LHY suppress *ELF4* expression. (a,b) Bioluminescence assays showing that *ELF4::LUC* expression was reduced in *fhy3 far1* (a) and *hy5 hyh* (b) mutants. Wild-type controls; No-0, Nossen ecotype; Ws, Wassilewskija ecotype. (c) Bioluminescence assays showing that the amplitude and rhythmic pattern of *ELF4* expression were altered by mutations in specific *cis* elements (*FBSm::LUC* and *ACEm::LUC*). c.p.s., counts per second. Data are means \pm s.d., $n = 25$. Data are representative of four independent lines. (d) qRT-PCR

assays showing that *ELF4* expression is reduced in the *CCA1-OX* transgenic plants. The white and grey bars in each panel indicate the subjective day and night, respectively (mean \pm s.d., $n = 3$). LL, continuous white-light conditions. Col-0; Columbia ecotype, wild type. (e,f) Transient expression assays showing that FHY3, FAR1 and HY5 upregulate (e), whereas CCA1 and LHY downregulate (f), *ELF4* expression in *Nicotiana benthamiana* leaves. CK, empty vector control. Relative expression of *ELF4::LUC* was normalized to *35S::REN* (internal control) (LUC/REN, mean \pm s.d., $n = 3$).

present in the same fractions of protein complexes *in vivo* at dawn (Supplementary Fig. S3b).

To test the effects of protein–protein interactions among FHY3, FAR1, CCA1, LHY and HY5 on their transcriptional activities, we carried out transient expression assays in *Nicotiana benthamiana* leaves. Co-infiltration with different effectors revealed that CCA1 and LHY not only directly suppressed the expression of the *ELF4::LUC* reporter gene by themselves, but also suppressed the expression of *ELF4::LUC* when co-expressed with FHY3, FAR1 or HY5 (Fig. 4d). In addition, co-expression of FHY3, FAR1 or HY5 with a *LUC* reporter gene driven by the *ELF4* promoter containing mutations in the EE elements (*ELF4mEE::LUC*) revealed that CCA1 and LHY could still partially suppress the transcriptional activation activity of FHY3 (Supplementary Fig. S4a), indicating that suppression of FHY3-promoted activation of *ELF4* expression by CCA1 and LHY does not require direct binding of CCA1 and LHY to the EE elements in the *ELF4* promoter. Moreover, EMSA analysis revealed that increasing protein amounts of CCA1 or LHY reduced the binding of FHY3 to the FBS-containing probe fragment (Supplementary Fig. S4b). Furthermore, a ChIP–qPCR assay revealed that the DNA-binding activity of FHY3 to the *ELF4* promoter was decreased at dusk in *CCA1-OX* (ref. 19) transgenic plants, compared with wild-type plants (Fig. 4e). Collectively, these results indicate that CCA1 and LHY suppress the transcriptional activation activity of FHY3 on the *ELF4* promoter at least partially through reducing the DNA-binding activity of FHY3 *in vivo*. Consistent with our observations, a previous study showed that CCA1 alters the DNA-binding activity of HY5 to the *Lhcb1*3* (*CAB1*) promoter through physical protein–protein interaction²².

It has been well documented that both the messenger RNA and protein levels of CCA1 and LHY are expressed at dawn and are regulated by the circadian clock^{19,23}. qRT–PCR assays showed that mRNA expression of *FHY3* and *FAR1* is not robustly regulated by the circadian clock under circadian conditions (Fig. 5a); however, bioluminescence analysis of the *FHY3::FHY3-LUC* fusion protein reporter gene (Supplementary Fig. S5) showed that the FHY3 protein level increases during the day and decreases at night under diurnal light–dark conditions (Fig. 5b). In addition, the FHY3 protein level is also regulated by the circadian clock (Fig. 5c). FHY3 protein accumulation peaks at dusk under both diurnal and circadian conditions, correlating well with the hypothesis that FHY3 is a direct positive regulator of *ELF4* expression and the demonstrated role of FHY3 in gating light signals to the clock¹³. Furthermore, there is no significant diurnal change for HY5 protein expression; however, HY5 is physiologically less active in the dark¹⁷, implying that HY5 promotes *ELF4* expression largely during the day.

Thus, we concluded that FHY3, FAR1 and HY5 define a set of constant positive transcription factors activating *ELF4* expression during the day, whereas CCA1 and LHY suppress the transcriptional activation activities of FHY3, FAR1 and HY5 periodically through inhibition of the DNA-binding activity of FHY3 (and possibly FAR1 and HY5 as well), with the strongest effect at dawn. This hypothesis is consistent with the observation that stronger binding of FHY3, FAR1 and HY5 to the *ELF4* promoter occurred at dusk, compared with that at dawn (Fig. 2b). At dusk, CCA1 and LHY protein accumulation is diminished, resulting in peaked expression of *ELF4* (Fig. 5d). Thus, the circadian-clock and diurnal regulation of FHY3 protein accumulation, and light regulation of HY5 protein activity could

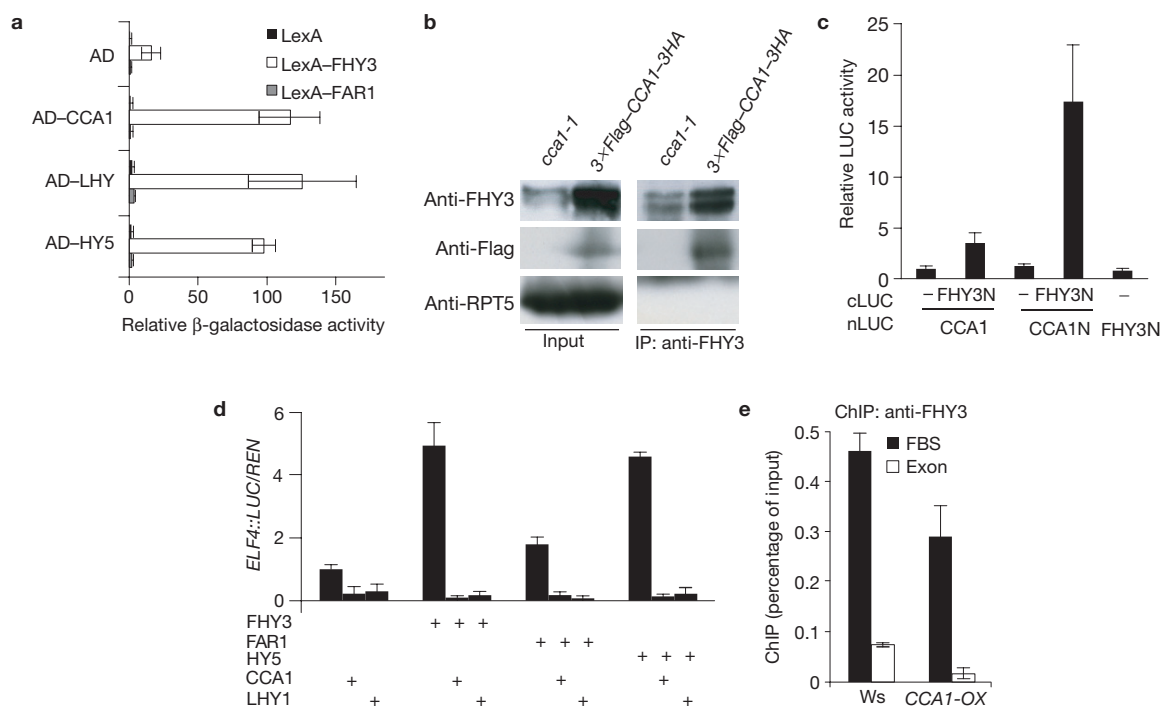


Figure 4 CCA1 and LHY suppress the transcriptional activation activity of FHY3, FAR1 and HY5 through direct physical interaction. **(a)** Yeast two-hybrid assays showing that FHY3, but not FAR1, interacts with CCA1, LHY and HY5 *in vitro* (mean \pm s.d., $n = 5$). **(b)** Co-immunoprecipitation assays showing that CCA1 specifically associates with FHY3 *in vivo*. Anti-RPT5 antibodies were used as a negative control for the immunoprecipitation experiment. **(c)** LCI assays showing that the N termini of FHY3 and CCA1 mediate the interaction. nLUC and cLUC, N-terminal or C-terminal fragment of firefly luciferase, respectively;

FHY3N and CCA1N, N-terminal fragment of FHY3 and CCA1, respectively. **(d)** CCA1 and LHY suppress the activation activity of FHY3, FAR1 and HY5 on *ELF4* expression in *Nicotiana benthamiana* leaves. Relative expression of *ELF4::LUC* was normalized to *35S::REN* (internal control; LUC/REN, mean \pm s.d., $n = 3$). **(e)** ChIP-qPCR assays showing that the increasing CCA1 protein level at ZT12 in *CCA1-OX* plants suppresses the binding of FHY3 to the *ELF4* promoter fragment containing the FBS *cis* element. An exon region of *ELF4* was used as a negative control in the ChIP-qPCR assay (mean \pm s.d., $n = 3$).

collectively contribute to the generation and maintenance of an accurate and robust *ELF4* rhythm. Consistent with this model, robust cycling of *ELF4* is not detectable under continuous darkness⁹, just as in the *fly3 far1* double mutants in the light, indicating that FHY3 and FAR1 are essential for the daily synchronization of *ELF4* expression by the light–dark cycle. In further support of this model, we found that the expression of *ELF4* was higher and peaked earlier at dawn (Zeitgeber time 4, ZT4) in a *cca1 lhy* double-mutant background²⁴, compared with wild-type plants under both diurnal light–dark and continuous white-light conditions (Fig. 5e).

Genome-wide analysis identified a set of 602 putative direct target genes of FHY3 and FAR1 (ref. 12) and about 30% of them (202 genes) have been documented to exhibit cycling expression in diurnal and/or circadian conditions; with 12% of them showing cycling expression in most tested diurnal or circadian conditions (Supplementary Table S1). Consistent with this, putative FHY3–FAR1-binding sites are highly over-represented in the promoters of these cycling genes (Supplementary Fig. S6a). Furthermore, genome-wide analysis revealed that the promoters of a group of well-known circadian-clock-regulated genes contain the putative binding sites for FHY3, FAR1, HY5, CCA1 and LHY, indicating that these genes may also be co-regulated by multiple transcription factors at the transcriptional level (Supplementary Table S2). In addition, the ChIP assay confirmed that FHY3 directly binds to the promoters of several circadian- and light-responsive genes, including *CCA1*, *CHE*, *COP1* (Constitutive

Photomorphogenic 1), *PHYB* (Phytochrome B), *PARI* (Phytochrome Rapidly Regulated 1) and *EPR1* (Early Phytochrome Responsive 1; Supplementary Fig. S6b).

Our data demonstrate that coordinated action and interaction between constant positive transcription factors FHY3, FAR1 and HY5 and periodic negative transcription factors CCA1 and LHY through their distinct cognate *cis*-elements are essential for establishing and sustaining the robust oscillations of *ELF4* expression and proper clock function in *Arabidopsis*. This is in sharp contrast with the observations made in other eukaryotes (fungi, insects and mammals), where circadian rhythmicity of key clock genes is generated through the interaction of positive and negative regulatory proteins on a single *cis*-element sequence^{25,26}. Here we show that in plants, although the clock-controlled evening element is sufficient to confer evening-phased gene expression²⁷, these light-responsive FBS sites and ACE-containing elements in the *ELF4* promoter (and possibly other circadian genes) are required to form the positive constituent of the transcriptional circuitry. This feature is unique to plants, which adds an additional layer of regulation that allows direct input of light signals to the promoter of a central clock gene, thus directly synchronizing the expression of clock genes and optimizing plant growth and fitness to the daily changes in the ambient-light environments. It is known that FHY3, FAR1 and HY5 are all downstream intermediates of *phyA* signalling²⁸. In addition, *phyA* itself is essential for *ELF4* expression²⁹ and *phyA* protein directly associates with FHY3 *in vivo*³⁰. Thus, these phytochrome

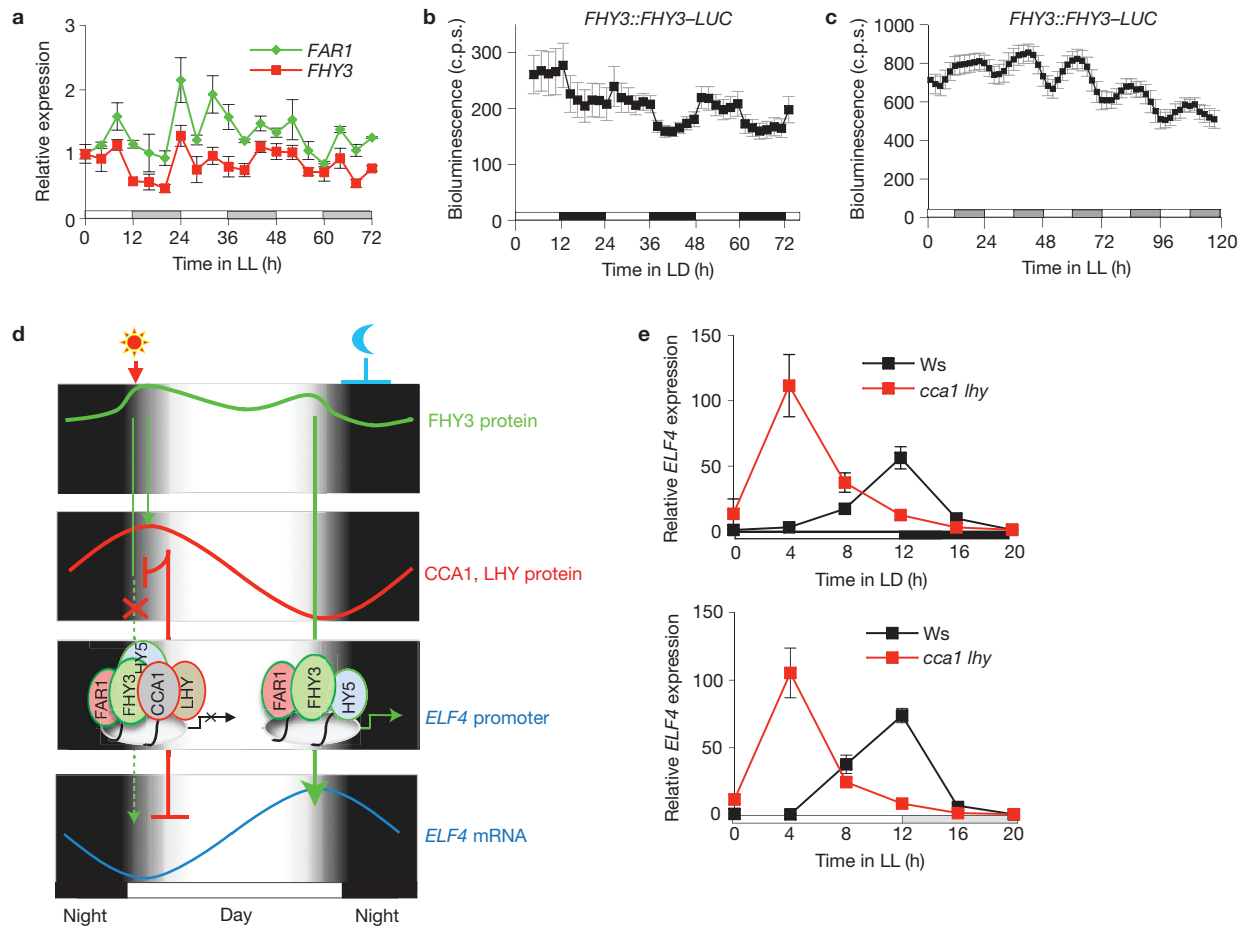


Figure 5 FHY3, FAR1, HY5, CCA1 and LHY coordinately regulate the cyclic expression of *ELF4*. **(a)** qRT-PCR analysis of *FHY3* and *FAR1* genes. *Arabidopsis* wild-type (No-0 ecotype) seedlings were entrained in cycles of 12 h light/12 h dark conditions for 7 days, and then released into continuous white-light conditions (LL) for 3 days. Expression of *FHY3* and *FAR1* is normalized to the expression of a *ubiquitin* gene. Data are means \pm s.d., $n = 3$. **(b, c)** Bioluminescence assays showing that the expression pattern of *FHY3::FHY3-LUC* is regulated by diurnal **(b)**; long day, LD) conditions and the circadian clock **(c)**. c.p.s., counts per second. Data are means \pm s.e.m., $n = 16$. **(d)** A model depicting the coordinated antagonistic regulation of *ELF4* expression by positively acting and negatively acting transcription

signalling molecules constitute a potential molecular link connecting environmental-light perception to the central oscillator. □

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>

Note: Supplementary Information is available on the Nature Cell Biology website

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factors at dawn and dusk. CCA1 and LHY can suppress *ELF4* expression by themselves or by suppressing the activation activities of FHY3 and FAR1. Arrow: positive regulation; bar: negative regulation; X: blocking effect. **(e)** qRT-PCR analyses showing that *ELF4* expression peaks higher and earlier in the *cca1 lhy* double-mutant background, compared with wild-type plants. *Arabidopsis* wild-type (Ws) and *cca1 lhy* double-mutant seedlings were entrained in cycles of 12 h light/12 h dark conditions for 4 days, and followed by 1 day of long-day (top panel) or continuous white-light (bottom panel) conditions. Expression of *ELF4* is normalized to the expression of an *actin* gene. The white and grey (or dark) bars at the bottom indicate the subjective light and dark period, respectively. Data are means \pm s.e.m., $n = 3$.

AUTHOR CONTRIBUTIONS

P.F.D., X.W.D., J.W. and H.W. contributed to project design. R.L. recorded the phenotype of *fly3* mutants and generated *FHY3-LUC* reporter lines. X.-Y.W. generated $3 \times \text{Flag-3} \times \text{HA}$ transgenic lines. H.S. carried out the bioluminescence analyses of all the *LUC* reporter lines. Y.T. carried out the yeast assays, qRT-PCR and generated *ELF4-OX* transgenic lines. G.L. carried out all other experiments. G.L., P.F.D. and H.W. wrote the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plant materials and growth conditions. The *fly3* alleles (*fly3-4*, *fly3-5*, *fly3-6*, *fly3-7*, *fly3-8*, *fly3-9* and *fly3-10*), *far1-2* and *fly3-4 far1-2* double mutant of *Arabidopsis* are in the No-0 ecotype³¹. *cca1-1* (ref. 32), *CCA1-OX* (ref. 19), *CCA1::GFP-CCA1* (ref. 8), *HY5::HY5-YFP* (ref. 18), *hy5 hyh* (ref. 33) and *cca1 lhy* (ref. 24) double mutants have been described previously. Luciferase reporter lines *CAB2::LUC* (ref. 34), *TOC1::LUC* (ref. 35) and *CAT3::LUC* (ref. 36) were introduced into *fly3-4 far1-2* mutants and their respective wild-type background (No-0 ecotype) through genetic crosses.

The plant growth conditions and the light source of far-red light were described previously³⁷. For the flowering-phenotype analysis, the plants were germinated and grown in soil under long-day (16 h light/8 h dark) or short-day (8 h light/16 h dark) conditions. Days to flowering and the numbers of visible rosette leaves (>2 mm) were recorded when the first flower bud opened. For the circadian-rhythm-related experiments, *Arabidopsis* seedling were entrained to cycles of 12 h light/12 h dark conditions for 7 days and then released into continuous white-light conditions for different durations.

Plasmid construction and generation of transgenic *Arabidopsis* plants. To generate *ELF4::LUC* reporter genes, a fragment containing the *ELF4* promoter (1,600 base pairs upstream of the ATG) was PCR amplified from *Arabidopsis* (Col-0) genomic DNA, and then the PCR fragments were inserted into *yy96* vector³⁸ digested with PstI and BamHI to produce *yy96-ELF4-LUC* vectors. Then, *yy96-ELF4-LUC* vector was used as the template to produce mutated *ELF4::LUC* (FBSm, ACEm) reporter genes according to the manufacturer's instructions for the QuikChange Site-Directed Mutagenesis Kit (Stratagene, 200518). Finally, the fragments containing various expression cassettes of *ELF4-LUC* were released from *yy96-ELF4-LUC* and inserted into pPZP211 or pPZP221 to produce pPZP211-*ELF4::LUC* or pPZP221-*ELF4::LUC*, respectively. The binary vector pPZP221-*ELF4::LUC* was transformed into *hy5 hyh* double-mutant and wild-type (Ws ecotype) backgrounds. The various constructs of pPZP211-*ELF4::LUC* were transformed into Col-0 wild-type, *fly3 far1* and No-0 wild-type backgrounds. Over 30 independent T1 transgenic lines for each construct were selected and transplanted into soil. The luciferase activities of T2 generation were detected using a Xenogen IVIS imaging system (Caliper) and then ten representative independent lines were used to carry out the bioluminescence analysis using a NightOwl Cooled CCD (charge-coupled device) camera (Berthold Technologies).

The fragment containing the firefly luciferase gene (Promega) was inserted into pPZP-FHY3p::FHY3 (ref. 39) digested with BamHI and SacI to produce pPZP-FHY3::FHY3-LUC and transformed into the *fly3-4* mutant. Two representative independent transgenic lines showing a complete rescue of the *fly3* mutant phenotype were selected for bioluminescence analysis. To generate the *ELF4-OX* expression construct, the coding sequence of *ELF4* was PCR amplified from the Col wild type and inserted into pSAT6-EYFP-C1 (ref. 40) digested with BglII and SalI to produce pSAT6-EYFP-C1-*ELF4*. Then, the expression cassette of 35Spro::EYFP-*ELF4* was released and inserted into pRCS2-OCS-Bar (ref. 40) digested with PI-PspI to produce pRCS2-EYFP-*ELF4*, which was then transformed into *fly3-4* mutants. To generate the 3 × Flag-CCA1-3 × HA expression construct, the oligonucleotide primers of 3 × Flag and 3 × HA were annealed and then the double-stranded oligonucleotides were digested with BglII and EcoRI, SalI and KpnI, respectively. The full-length coding sequence of CCA1 was amplified by RT-PCR from Col-0 and then digested with EcoRI and SalI. Then, the fragments of 3 × Flag, CCA1 and 3 × HA were ligated together and inserted into pSAT6-MCS (ref. 40) vector digested with BglII and KpnI to produce pSAT6-3 × Flag-CCA1-3 × HA. Then the expression cassette of 35Spro:: 3 × Flag-CCA1-3 × HA was released from pSAT6-3 × Flag-CCA1-3 × HA by PI-PspI digestion and then inserted into PI-PspI-digested pRCS2-OCS-Bar (ref. 40) to produce pRCS2-3 × Flag-CCA1-x3 × HA. pRCS2-3 × Flag-FHY3-3 × HA was generated using a similar strategy, except MfeI, instead of EcoRI, was used to digest PCR product containing the full-length coding sequence of FHY3. To generate the pSPY-FAR1::FAR1-Myc expression construct, the fragment containing the coding sequence of FAR1 was inserted into the pSPYNE vector digested with XbaI and BamHI to produce pSPYNE-FAR1-Myc. Then, the fragment containing the FAR1 promoter was inserted into pSPYNE-FAR1 digested with HindIII and XbaI to produce pSPY-FAR1::FAR1-Myc. Finally, these binary vectors were transformed into *cca1-1*, *fly3-4* or *far1-2* mutants, respectively. Transgenic plant screening and identification were described previously¹². Over 30 independent transgenic lines of the T1 generation were selected and transplanted into soil. Expression of the fusion proteins in T2 plants was detected using western blotting. T3 homozygous lines were used to carry out further experiments.

qRT-PCR. The RNA extraction and qRT-PCR assay procedures were described previously¹². *Arabidopsis* seedlings were grown in cycles of 12 h light/12 h dark conditions for 7 days and then released into continuous light conditions. The samples were collected every 4 h after release into continuous light conditions.

The primers for detecting *CCA1*, *LHY*, *ELF4* and *Ubiquitin (UBQ)* expression were described previously^{11,12}.

Transient expression assay. Transient expression assays (Dual-LUC) were carried out as described previously^{41,42}. To generate pGreen-*ELF4::LUC*, the full-length *ELF4* promoter was released from *yy96-ELFP1-LUC* by PstI and BamHI digestion and then inserted into pGreen-0800-LUC (ref. 41) to produce pGreen-*ELF4-LUC*. To generate SPYNE-FHY3, SPYNE-CCA1, SPYNE-LHY and SPYNE-HY5, the fragments containing the full-length FHY3, CCA1, LHY and HY5 coding sequences were inserted into SPYNE (ref. 43) digested with BamHI and SalI. To produce SPYNE-FAR1, the fragments containing the full-length FAR1 coding sequences were inserted into BamHI- and XhoI-digested SPYNE (ref. 43). Then, the Luciferase reporter (pGreen-*ELF4::LUC*, or pGreen-*ELF4mEE::LUC*) and various effector (SPYNE) constructs were introduced into *Agrobacterium* strain C58C1 to carry out co-infiltration in *Nicotiana benthamiana* leaves.

LCI assay. LCI assays were carried out as described previously⁴⁴. The fragments containing full-length CCA1 and the N terminus of CCA1 (1–338AA) were PCR amplified and inserted into pCambia1300-nLUC (ref. 44) digested with BamHI and SalI, to produce nLUC-CCA1 and nLUC-CCA1N, respectively. A KpnI/SalI fragment containing FHY3N was inserted into pCambia1300-cLUC to produce cLUC-FHY3N. Finally, various nLUC and cLUC constructs were introduced into *Agrobacterium* strain GV2260 to carry out co-infiltration in *Nicotiana benthamiana* leaves. Luciferase activities were detected using Xenogen IVIS Spectrum and quantified using Living Image software (Caliper).

Yeast assay. Yeast one-hybrid and two-hybrid assays were carried out as described previously^{12,31}. LexA-FHY3, LexA-FAR1, AD-FHY3 and AD-FAR1 have been described previously¹². To generate AD-CCA1, AD-LHY and AD-HY5, the full-length CCA1, LHY and HY5 coding sequences were PCR amplified by RT-PCR from the Col-0 wild-type background and ligated into the pB42AD vector (Clontech) digested with EcoRI and XhoI. To generate *ELF4p::LacZ* reporter genes, the various fragments of the *ELF4* promoter were amplified from *yy96-ELF4::LUC* and digested with EcoRI and KpnI, then inserted into pLacZi2μ (ref. 12) to generate *ELF4p::LacZ*.

EMSA. Glutathione S-transferase (GST), GST-FHY3N and GST-FAR1N recombinant fusion proteins were described previously¹². To generate GST-CCA1, GST-LHY and GST-HY5 constructs, the full-length coding sequences of CCA1, LHY and HY5 were inserted into pGEX4T-1 (GE Healthcare) digested with EcoRI and XhoI. Full-length CCA1 and LHY complementary DNAs were cloned into pET-28a, generating N-terminal histidine-T7-tagged fusions. The recombinant fusion protein expression and purification, and the procedure of EMSA were carried out as described previously¹². The oligonucleotide sequence of the probe containing FBS sites has been described previously¹². The oligonucleotide sequences of the other probes are listed in Supplementary Table S3.

ChIP assay. *Arabidopsis* seedlings were grown in 12 h light/12 h dark conditions for 7 days and the samples were collected at ZT1 and ZT12. The ChIP experiment was carried out following the procedure described previously¹⁷. Rabbit anti-FHY3 polyclonal antibodies³⁰, anti-HY5 polyclonal antibodies⁴⁵, anti-GFP polyclonal antibodies (Abcam, ab290), anti-c-Myc polyclonal antibodies (Sigma, A5598) and mouse anti-Flag (Sigma, A8592) antibodies were used at 1:1,000. The primers -400F and ATG-R (responsible for a fragment containing 400 base pairs upstream of ATG) in the *ELF4* promoter were used for ChIP-PCR. All of the primers used in ChIP-PCR or ChIP-qPCR are listed in Supplementary Table S3.

Immunoblot, immunoprecipitation and gel-filtration chromatography. The immunoprecipitation, gel-filtration and immunoblotting procedures were described previously³⁰. For co-immunoprecipitation, four-day-old *Arabidopsis* seedlings grown in long-day conditions were treated with 50 μM MG132 (Sigma, C2211) and 50 μM MG115 (Sigma, C6706) from ZT16 and collected at ZT25 the next day. Total proteins were extracted using IP lysis buffer (50 mM Tris-HCl, at pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 0.1% Triton X-100) with freshly added PMSF (phenylmethylsulphonyl fluoride, 2 mM), protease inhibitor cocktail (Roche, 11873580001), 50 μM MG132 and 50 μM MG115. Anti-FHY3 antibodies and anti-rabbit IgG agarose beads (Sigma, A8914) were used for the immunoprecipitation. Anti-RPT5 (Regulatory Particle 5a of 26S proteasome) antibodies⁴⁶ were used as a loading control. For gel-filtration chromatography, seven-day-old *Arabidopsis* seedlings grown in long-day conditions were collected at ZT1. The seedlings were homogenized in IP lysis buffer with freshly added PMSF and protease inhibitor cocktail. Mouse anti-Flag (M2, sigma, A8592) and rabbit anti-LHY antibodies⁴⁷ were used to detect the 3 × Flag-FHY3-3 × HA fusion protein and endogenous LHY protein, respectively. The dilution for all antibodies was 1:1,000.

Primers. All of the primers used in this study are listed in Supplementary Table S3. The primers used to detect *CCA1*, *LHY*, *ELF4* and *Ubiquitin* expression in qRT-PCR and mutagenesis of FBS *cis* elements were described previously^{11,12}.

Accession numbers. Sequence data from this Letter can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *FHY3* (At3g22170), *FAR1* (At4g15090), *CCA1* (At2g46830), *LHY* (At1g01060), *HY5* (At5g11260), *ELF4* (At4g20080), *actin* (At3g46520) and *Ubiquitin* (At3g52590).

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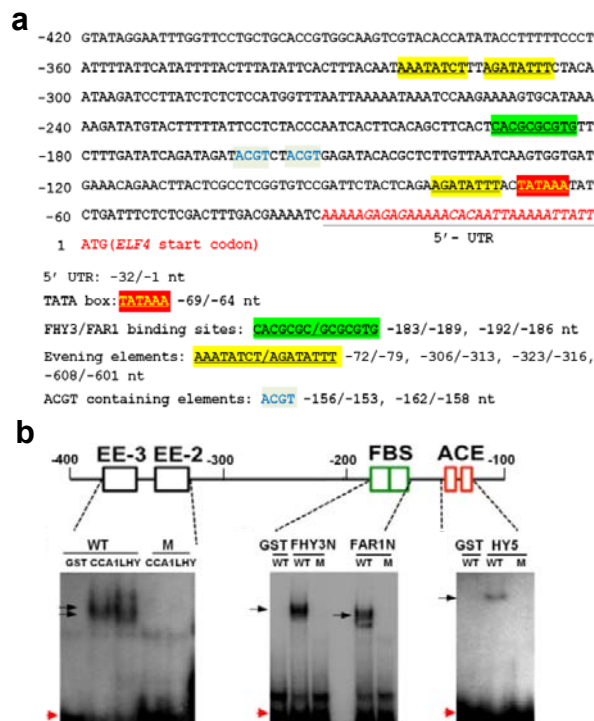


Figure S1 FHY3, FAR1, HY5, CCA1 and LHY directly bind to the *ELF4* promoter *in vitro*. **a**, Nucleotide sequence of the proximal region of *ELF4* promoter. The start codon of *ELF4* (ATG) is shown at the end. 5'-UTR, TATA box, and various *cis*-elements are labeled. **b**, EMSA assay showing that FHY3, FAR1, CCA1, LHY and HY5 bind to the specific *cis*-elements in the *ELF4* promoter *in vitro*. Top panel, A diagram showing the distribution of the

various *cis*-elements in the *ELF4* promoter. The regions between the dashed lines mark the specific probes used in the EMSA assays. The black arrows indicate the positions of protein-DNA complexes. The red arrows indicate the free-probes. WT: wild type probes; M: mutant probes. The oligonucleotide sequences of the WT and mutant probes are listed in the Supplementary Table 3.

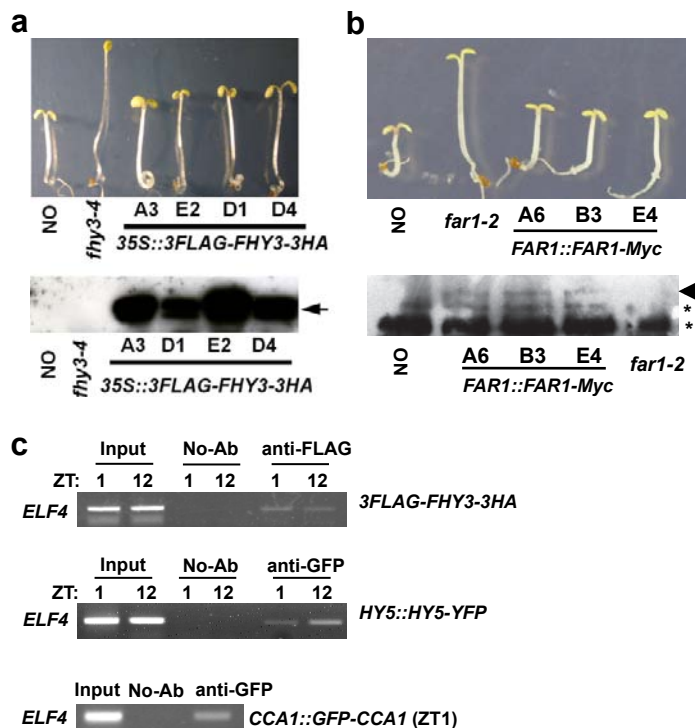


Figure S2 FHY3, HY5 and CCA1 directly bind to the *ELF4* promoter *in vitro*. **a-b**, Phenotypic and molecular characterization of *3FLAG-FHY3-3HA* and *FAR1::FAR1-Myc* transgenic plants. Top panels, overexpression of *3FLAG-FHY3-3HA* or *FAR1-Myc* fusion protein largely rescued the long-hypocotyl phenotype of the *fhy3-4* or *far1-2* mutant under continuous far-red light conditions, suggesting that the fusion protein is biologically functional. A3, E2, D1 and D4 are four independent transgenic lines of *35S::3FLAG-FHY3-3HA*. A6, B3 and E4 are three independent transgenic lines of *FAR1::FAR1-Myc*. Bottom panels, western blotting showing the accumulation of *3FLAG-FHY3-3HA* or *FAR1-Myc* fusion protein (indicated

by arrows). Asterisks indicate cross-reactive bands. Anti-FLAG antibodies (clone M2, Sigma, A8592) or anti-Myc antibodies (sigma, A5598) were used to detect the fusion proteins. **c**, Chromatin immunoprecipitation PCR assays showing that the *ELF4* promoter fragments containing the putative FHY3, HY5 and CCA1 binding sites are specifically enriched. *3FLAG-FHY3-3HA*, *HY5::HY5-YFP* and *CCA1::GFP-CCA1* were used in the ChIP assay. Anti-FLAG or anti-GFP antibodies were used to perform immunoprecipitation assay. An fragment containing the proximal 400bp upstream of ATG start codon of *ELF4* was used to perform the ChIP-PCR assay.

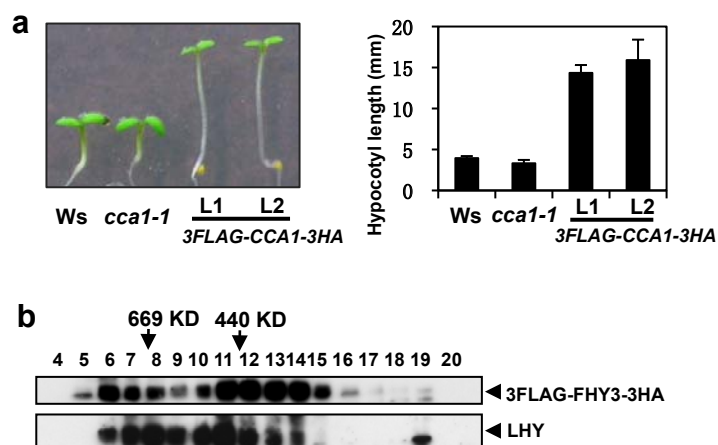


Figure S3 CCA1 and LHY direct interact with FHY3. **a**, Characterization of the *35S::3FLAG-CCA1-3HA* transgenic plants in the *cca1-1* mutant background. Overexpression of the *3FLAG-CCA1-3HA* fusion gene induced a long-hypocotyl phenotype under 12 h light-12 h darkness conditions (7-day-old seedlings were shown). The long-hypocotyl and delayed flowering (data not shown) phenotype of *3FLAG-CCA1-3HA* transgenic plants is consistent with a previous report¹. Data are means \pm s.d., $n > 10$. **b**, Gel-filtration

chromatography assay showing that the endogenous LHY protein co-filtrates with the 3FLAG-FHY3-3HA fusion protein in *Arabidopsis*. Anti-FLAG and anti-LHY antibodies were used to detect the 3FLAG-FHY3-3HA and LHY proteins in the *35S::3FLAG-FHY3-3HA* transgenic plants. Fractions 4 to 20 (of a total of 26 collected fractions) were subject to western blot analysis. The seedlings were grown under 12h light-12h darkness conditions for 7 days and harvested at ZT1 for protein extraction.

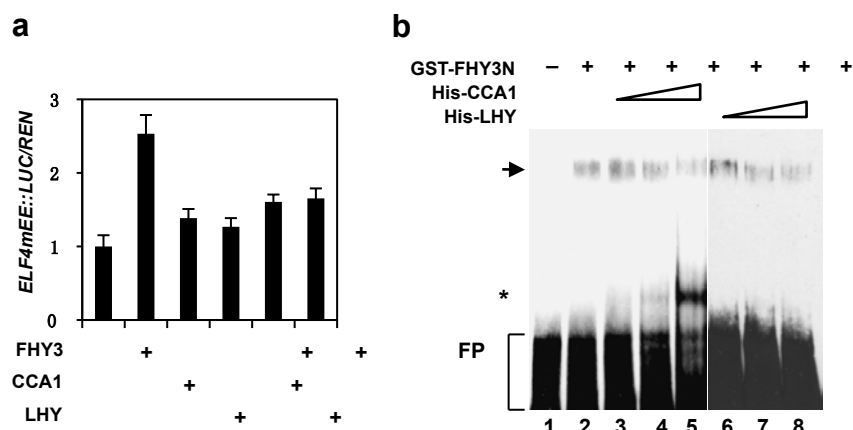


Figure S4 CCA1 and LHY suppress the transcriptional activation activity of FHY3. **a**, Transient expression assays showing that suppression of the transcriptional activation activity of FHY3 by CCA1 and LHY does not require their binding to DNA. An *ELF4mEE::LUC* reporter gene (EE elements were mutated in the *ELF4* promoter) was used to perform the transient expression assay. Relative expression of *ELF4mEE::LUC* was normalized to *35S::REN* (internal control) (mean ± s.e.m., $n=3$). **b**, EMSA analysis showing that increasing amounts of His-CCA1 or His-LHY proteins

directly suppress FHY3 binding to the *ELF4* promoter. Arrow indicates protein-DNA complex of GST-FHY3N protein and the *ELF4* probe. The open triangles indicate increasing protein amounts (0.5, 1, 2.5 μg) of His-CCA1 (lanes 3-5) and His-LHY (lanes 6-8) fusion proteins. 0.5 μg of GST-FHY3N recombination protein was used in lanes 2 to 8. GST alone was added in lane 1. An fragment containing the FBS sites in the *ELF4* promoter was used as the probe. FP, free probe. Asterisk indicates a non-specific band.

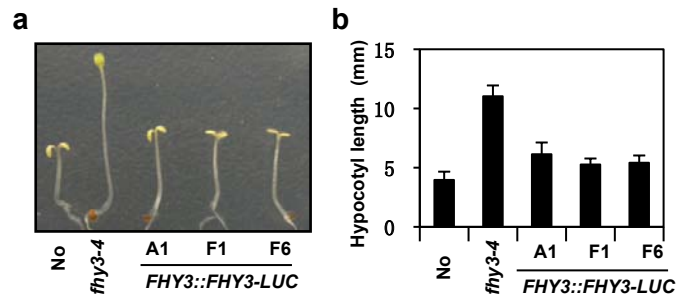


Figure S5 Phenotypic and molecular characterization of *FHY3::FHY3-LUC* transgenic plants in the *fhy3-4* mutant background. The *FHY3::FHY3-LUC* fusion gene rescued the long-hypocotyl phenotype of the *fhy3-4* mutant

under continuous far-red light conditions (4-day old seedlings were shown), suggesting that the FHY3-LUC fusion protein is biologically functional. Data are means \pm s.d., n=10.

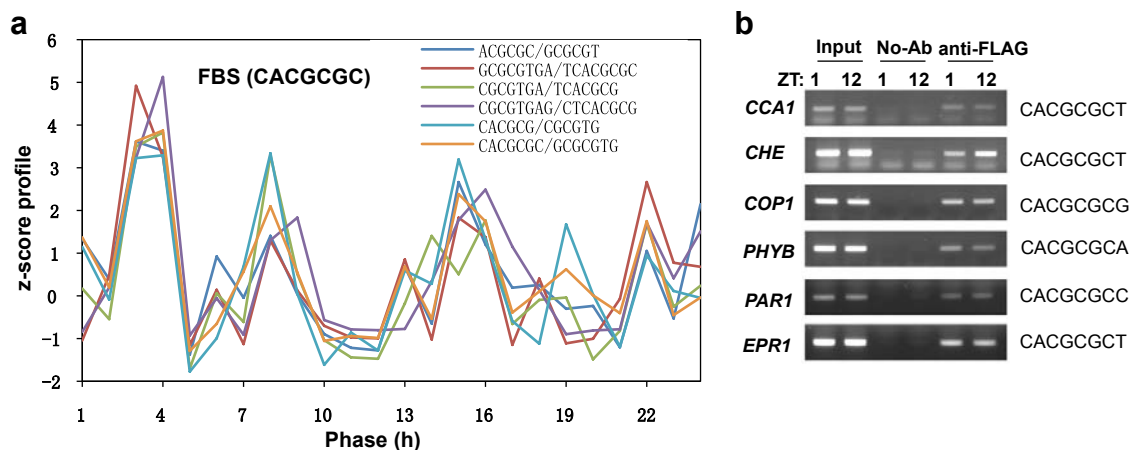


Figure S6 Genome-wide identification of the overrepresented FBS elements over the day under continuous light conditions. **a**, Z-score profiles of overrepresented 6-8mer words composing the FBS elements over the day under continuous light conditions. Putative FBS cis-elements are highly overrepresented in the ZT3 to ZT4 ranges (LL), which is consistent with the observation that FHY3 protein accumulation is rapidly induced by light. The minor Z score peaks at various time points of the day suggest that the FBS motifs might be important over a broad range of phases during the day-night cycle, or the whole pattern might be noisy (5%

FDR). Z-score profiles of FBS *cis*-elements were extracted from previous microarray data² (LL-LDHH dataset). **b**, ChIP-PCR analysis showing that FHY3 directly bind to the promoters of several circadian clock regulated genes, including *CCA1*, *CHE*, *COP1*, *PHYB*, *PAR1* and *EPR1*. The DNA sequence of the putative FBS site in the promoter of each target gene is shown on the right. 7-day old *35S::3FLAG-FHY3-3HA* seedlings grown in 12 h light-12 h darkness conditions were harvested at ZT1 and ZT12. Anti-FLAG antibodies were used for the ChIP assay. No-Ab, no-antibodies immunoprecipitation control.

Figure 4b

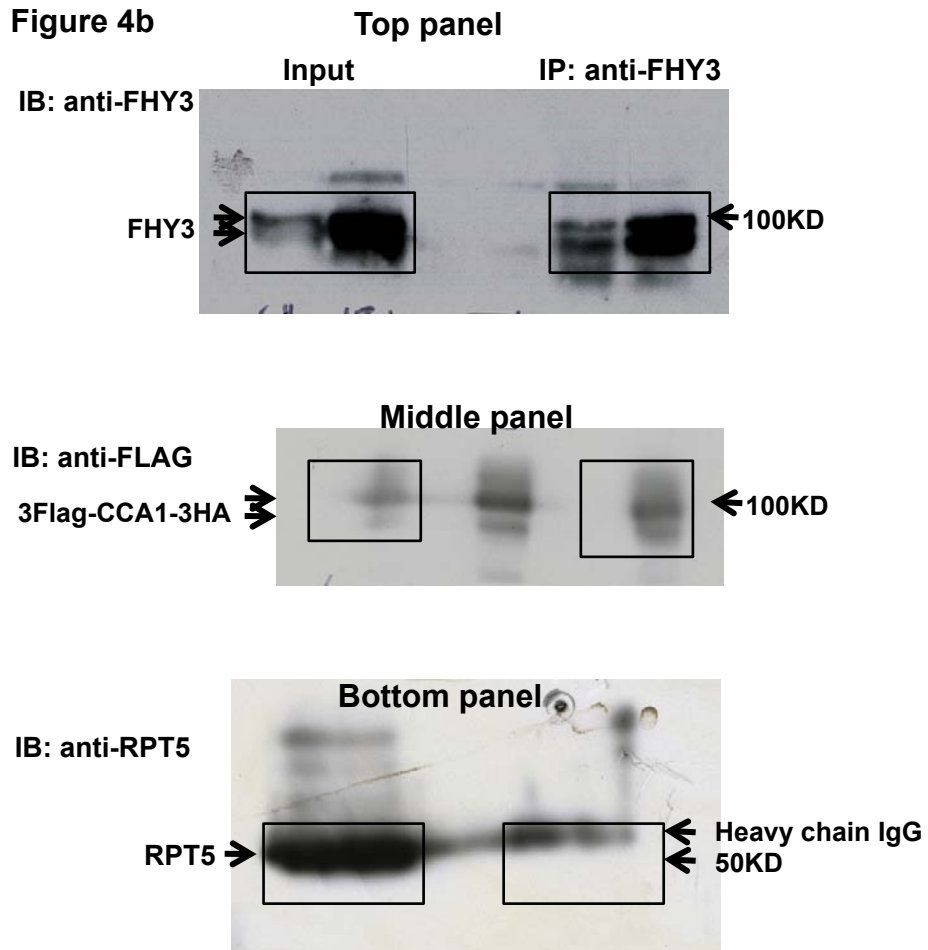


Figure S7 Full scans

Supplementary Tables

Supplementary Table 1. Genome wide analysis of putative direct target genes of FHY3 and FAR1.

A set of 602 putative direct target genes of FHY3 and FAR1 were identified from the *Arabidopsis* genome based on the existence of FBS in the upstream (500 bp) of putative transcriptional start site of each gene (TAIR 9) using Patmatch analysis (<http://www.arabidopsis.org>). About 30% of putative targets of FHY3 (totally 202, as listed) exhibits cycling expression in at least one of the tested circadian or diurnal conditions; with 12% putative targets of FHY3 (totally 74, filled with yellow color) showing cycling expression in most tested circadian or diurnal conditions (<http://diurnal.cgrb.oregonstate.edu>, correlation >0.8).

Locus	FBS	EE	CBS	Gene Description
AT2G46830	120 R			Circadian clock associated 1 (CCA1)
AT2G40080	340 F, 343 R	209 F 219 R 453 R		Early flowering 4 (ELF4)
AT5G37260	56 F		140 F	Circadian 1 (CIR1), Reveille 2 (RVE2)
AT5G08330	387 R			CCA1 hiking expedition (CHE)
AT3G07650	219 R	7 R		Constans like 9 (COL9)
AT2G32950	366 F			Constitutive photomorphogenic 1 (COPI)
AT2G18790	207 F			Phytochrome B (PHYB)
AT1G18330	301 R	199 F	410 F	Early phytochrome responsive 1 (EPR1)
AT2G42870	59 R			Phytochrome rapidly regulated 1 (PAR1)
AT3G58850	122 R			Phytochrome rapidly regulated 2 (PAR2)
AT3G56090	386 F	349 F		Ferritin 3
AT2G43330	230 F	342 R		Inositol transporter 1 (INT1)
AT3G58570	334 F	488 F, 147 R		Putative DEAD box RNA helicase
AT5G48250	351 R, 30 R	55 F 373 F 191 R 155 R		Unknown protein,
AT5G63880	184 F	468 R		Vesicle-mediated transport (VPS 20.1)
AT5G57110	372 R		313 R	Autoinhibited Ca ²⁺ -ATPase, isoform 8 (ACA8)
AT5G67480	284 F, 243 R	343 F		BTB and TAZ domain protein 4
AT5G03140	357 F		183 F	Lectin protein kinase family protein
AT5G14260	269 R		441 R	SET domain-containing protein
AT4G36930	435 R		62 F	SPATULA (SPT), bHLH type transcription factor
AT2G42190	373 F		179 R	Unknown protein
AT2G25250	355 R		460 F	Unknown protein
AT3G19720	455 R			Accumulation and replication of chloroplast 5

AT1G76990	100 F, 315 F			ACT domain repeat 3 (ACR3)
AT1G27450	97 R			Adenosine phosphoribosyl transferase
AT3G23920	364 F			Chloroplast beta-amylase (BAm1)
AT1G09340	170 R			Chloroplast RNA binding (CRB)
AT4G26700	434 R			Fimbrin-like protein
AT2G45160	131 F			Hairy meristem 1 (HAM1)
AT4G00150	383 F, 386 R			Hairy meristem 3 (HAM3)
AT4G30350	468 F			Heat shock protein-related
AT1G01490	380 F, 394 F			Heavy-metal-associated domain-containing protein
AT5G05320	427 F			Monoxygenase, putative (MO3)
AT2G22240	181 F			Myo-inositol-1-phosphate synthase isoform 2
AT3G56480	409 R			Myosin heavy chain-related
AT3G55960	308 R			NLI interacting factor (NIF) family protein
AT5G47640	219 F			Nuclear factory Y, subunit B2 (NF-YB2)
AT1G56170	300 F			Nuclear factory Y, subunit C2 (NF-YC2)
AT5G63620	482 R			Zinc-binding dehydrogenase family protein
AT5G62130	109 F, 448 F			Per1-like protein
AT5G64120	355 F			Peroxidase
AT3G61070	348 F			Peroxin11 (PEX11)
AT4G28610	422 F			Phosphate starvation response 1 (PHR1)
AT1G73980	44 R			Phosphoribulokinase uridine kinase family protein
AT3G46780	60 R			Plastid transcriptionally active 16 (PTAC16)
AT1G69730	27 F			Protein kinase family protein
AT2G45590	111 F			Protein kinase family protein
AT4G02630	241 F			protein kinase family protein
AT3G06500	415 F			Putative beta-fructofuranosidase
AT3G14620	21 F			Putative cytochrome P450 (CYP72A8)
AT1G23820	297 R			Spermidine synthase (SPDS1)
AT4G03390	154 F			Strubbelig receptor family 3 (SRF3)
AT1G70610	379 F			Transporter associated with antigen processing 1
AT2G18700	179 F			Trehalose synthase like domain containing protein
AT4G38960	387 R			zinc finger (B-box type) family protein
AT2G24100	438 R			Unknown protein
AT2G46735	277 F			Unknown protein
AT3G01060	188 F			Unknown protein
AT3G06080	12 F , 92 R			Unknown protein
AT3G12320	436 F , 452 F			Unknown protein
AT5G67370	162 F			Unknown protein
AT5G12050	444 F			Unknown protein
AT5G48470	364 R			Unknown protein
AT5G59400	400 F			Unknown protein
AT5G10695	8 R			Unknown protein
AT4G09970	138R 116R			Unknown protein

AT4G09970	138R 116R 89R			Unknown protein
AT5G12470	426 F			Unknown protein
AT5G11740	207 F			Arabinogalactan protein (AGP15).
AT2G45720	199 F			Armadillo beta-catenin repeat family protein
AT1G01650	413 F			Aspartic-type endopeptidase peptidase
AT2G37630	314 F			Asymmetric leaves 1 (AS1)
AT4G04970	155 F			Callose synthase like 1 (GSL1)
AT2G28800	449 F			Chloroplast membrane protein ALBINO 3 (ALB3)
AT1G31800	306 R, 264 R			Cytochrome P450 type monooxygenase 97A3
AT5G65110	202 F		343 F	Acyl-CoA oxidase 2 (ACX2)
AT2G41460	410 R		205 R	Apurinic endonuclease-redox protein (ARP)
AT3G19350	347 R	425 R	40 F	Maternally expressed PAB c terminal (MPC)
AT3G03380	45 R	376 R		Putative DegP protease 7 (DegP7)
AT3G56710	368 F	325 R		SIGMA factor binding protein 1 (SIB1)
AT3G04120	310 R	74 F		Cytosolic GADPH (C subunit)
AT2G45850	351 F, 178 R	62 R	9 F	DNA-binding family protein
AT4G34200	388 F, 404 R		192 F	Embryo sac development arrest 9 (EDA9)
AT1G74390	447 R		420 R	Exonuclease family protein
AT4G21810	375 F		311 R	DERLIN-2.1 (DER2.1)
AT1G68060	233 F		17 R	Microtubule associated protein (MAP70-1)
AT3G49530	440 F		161 F	NAC domain containing protein 62
AT1G73710	382 F		421 R	Pentatricopeptide (PPR) repeat-containing protein
AT3G13226	458 F, 485 F		132 R	Regulatory protein RecX family protein
AT5G67300	206 F, 220 R	80 R		Myb protein 44
AT3G01210	415 F	351 R		Nucleic acid binding oxidoreductase
AT5G65440	299 F		132F 200F	Unknown protein
AT1G32920	354 R		344 F	Unknown protein
AT1G01140	444 R			CBL-interacting protein kinase 9
AT3G27850	407 F			50S ribosomal protein L12-C
AT1G76730	233 F, 288 R			5-formyltetrahydrofolate cyclo-ligase family protein
AT4G18950	473 F			Ankyrin protein kinase
AT4G26740	412 R			Arabidopsis thaliana seed gene 1 (ATS1)
AT1G20900	29 F			AT hook domain containing protein
AT3G47640	440 F			Basic helix-loop-helix (bHLH) family protein
AT3G06670	360 R			Binding
AT3G47080	383 F			Binding
AT5G10730	411 F			Binding catalytic coenzyme binding
AT3G59020	438 F			Binding protein transporter
AT3G01770	276 R			Bromodomain and extraterminal domain protein 10
AT2G40950	410 F			bZIP17
AT3G10800	436 F, 448 R			bZIP28
AT5G26600	371 F			Catalytic pyridoxal phosphate binding

AT3G01370	165 F			CRM family member 2 (AtCFM2)
AT5G14800	453 F			Delta 1-pyrroline-5-carboxylate reductase
AT4G04860	473 F			DERLIN-2.2 (DER2.2)
AT3G17770	128 R			Dihydroxyacetone kinase family protein
AT3G61260	122 F, 151 F			DNA-binding family protein
AT3G62800	72 F			Double stranded RNA binding protein 4 (DRB4)
AT1G34430	380 R			Embryo defective 3003 (EMB3003)
AT1G72280	271 F, 274 R			Endoplasmic reticulum oxidoreductins 1 (AERO1)
AT1G32490	145 F			Enhanced silencing phenotype (ESP3)
AT1G19970	220 F			ER lumen protein retaining receptor family protein
AT2G47630	451 F			Esterase lipase, thioesterase family protein
AT4G02350	270 F			Exocyst complex component SEC15B (SEC15B)
AT4G08685	483 F			Expressed protein(SAH7)
AT2G03240	207 F			EXS family protein
AT1G31930	430 F, 326 R			Extra-large GTP-binding protein 3 (XLG3)
AT1G23020	354 R, 333 R			Ferric chelate reductase (FRO3)
AT2G40300	382 F			Ferritin 4
AT5G13840	415 F			Fizzy related 3 (FZR3)
AT5G10450	443 R			G-box regulating factor 6 (GRF6)
AT1G09560	25 F			Germin-like protein (GLP5)
AT1G71070	406 F, 348 R			Glycosyltransferase family 14 protein
AT2G30980	439 F			GSK3-like protein kinase
AT4G35380	316 F, 344 F			Guanine nucleotide exchange family protein
AT5G02490	262 F			Heat shock cognate 70 kDa protein 2 (HSC70-2)
AT1G63950	171 R			Heavy-metal-associated domain-containing protein
AT3G47810	296 R			Homolog of yeast retromer subunit VPS29
AT5G54680	461 R			Iaa-leucine resistant3 (ILR3)
AT4G23060	407 F			IQ-domain 22 (IQD22)
AT1G03310	392 R, 352 R			Isoamylase, debranching enzyme 1 (DBE1)
AT3G17860	189 F			Jasmonate-ZIM-domain protein 3 (JAZ3)
AT3G23750	482 R			Leucine-rich repeat (LRR) containing protein
AT3G02550	419 F			LOB domain containing protein 41 (LBD41)
AT5G47010	317 F			Low level beta amylase (LBA1)
AT3G59970	18 F			Methylenetetrahydrofolate reductase 1 (MTHFR1)
AT1G51660	404 F			Mitogen-activated map kinase kinase 4 (MKK4)
AT3G10760	237 F			Myb family transcription factor
AT4G39800	14 F			Myo-inositol-1-phosphate synthase isoform 1 (MIPS1)
AT5G13850	258 R			Nascent polypeptide complex subunit alpha like 3
AT5G67330	103 R			Natural resistance associated macrophage protein 4
AT3G60340	369 F			Palmitoyl protein thioesterase family protein
AT1G31790	304 F, 346 F			Pentatricopeptide (PPR) repeat-containing protein
AT3G28200	424 R			Peroxidase 31
AT3G60620	423 R			Phosphatidate cytidyltransferase

AT1G20380	319 F			Prolyl oligopeptidase
AT1G17145	437 R			Protein binding zinc ion binding
AT1G78420	420 R			Protein binding zinc ion binding
AT4G31170	324 R			protein kinase family protein
AT2G42500	456 F			Protein phosphatase 2A subunit (PP2A-4)
AT1G24170	425 R			Putative galacturonosyltransferase,
AT5G58720	436 R, 405 R			Putative PRL1-interacting factor
AT5G53570	445 F, 413 R			RabGAP TBC domain-containing protein
AT3G19630	31 F			Radical SAM domain-containing protein
AT3G07880	482 R			Rho GDP-dissociation inhibitor family protein
AT4G39100	391 F			Short life (SHL1)
AT2G41180	290 R			SigA-binding protein-related
AT5G67250	378 R			SKP1 interacting partner (SKIP2)
AT3G07590	222 F			Small nuclear ribonucleoprotein D1
AT3G27060	269 R			Small subunit of the 3 ribonucleotide reductase (RNR)
AT3G27380	271 F			Succinate dehydrogenase 2-1 (SDH2-1)
AT5G04590	280 F, 309 F			Sulfite reductase (SIR)
AT3G59770	189 R			Suppressor of actin 9 (SAC9)
AT2G23810	308 R			Tetraspanin 8 (TET8)
AT2G27860	388 F			UDP-d-apiiose UDP-d-xylose synthase
AT1G78270	196 R			UDP-glucosyl transferase 85A4 (AtUGT85A4)
AT1G78130	381 F			Unfertilized embryo sac 2 (UNE2)
AT4G32530	429 F, 444 R			vacuolar ATP synthase, putative V-ATPase
AT4G02620	486 R			vacuolar ATPase subunit F family protein
AT1G19910	440 F			Vacuolar H ⁺ -pumping ATPase 16 kDa proteolipid
AT3G13290	309 R			Varicose related (VCR)
AT1G15690	354 F			V-PPASE 3 (AVP3)
AT4G24120	411 R			Yellow stripe like 1 (YSL1)
AT5G62460	465 F			zinc finger (C3HC4-type RING finger) family protein
AT3G51950	397 R, 369 R			zinc finger (CCCH-type) family protein
AT5G12850	407 F			zinc finger (CCCH-type) family protein
AT1G68140	444 F			Unknown protein
AT1G13380	347 F, 405 F			Unknown protein
AT1G48300	55 R			Unknown protein
AT3G49590	156 R			Unknown protein
AT5G26850	481 F			Unknown protein
AT3G09180	158 R			Unknown protein
AT1G79510	490 F			Unknown protein
AT1G80040	398 F			Unknown protein
AT3G03160	19 R , 7 R			Unknown protein
AT3G07580	348 R			Unknown protein
AT3G07760	360 R			Unknown protein
AT3G16200	259 F			Unknown protein

AT3G17930	419 F			Unknown protein
AT3G21400	488 R			Unknown protein
AT4G38090	238 R			Unknown protein
AT4G01290	418 R, 393 R			Unknown protein
AT5G52200	336 F			Unknown protein
AT4G32020	380 R			Unknown protein
AT5G57080	361 F			Unknown protein
AT5G13760	298 F			Unknown protein

Supplementary Table 2. Genome wide analysis of putative direct target genes co-regulated by FHY3, FAR1, CCA1, LHY and HY5.

A set of 12 putative direct target genes of FHY3, FAR1, CCA1, LHY and HY5 were identified from the *Arabidopsis* genome based on supplemental table S1 and previous HY5 ChIP-chip analysis³. The exact locations of HY5 binding sites in the promoter of each putative direct target gene have not been identified. A set of direct target genes with cyclic expression in most tested circadian or diurnal conditions are filled with yellow color (<http://diurnal.cgrb.oregonstate.edu>, correlation >0.8).

Locus	FBS	EE	CBS	Gene Description
AT2G40080	340 F, 343 R	209 F 219 R 453 R		Early flowering 4 (ELF4)
AT5G37260	56 F		140 F	Circadian 1 (CIR1), Reveille 2 (RVE2)
AT3G07650	219 R	7 R		Constans like 9 (COL9)
AT1G18330	301 R	199 F	410 F	Early phytochrome responsive 1 (EPR1)
AT2G43330	230 F	342 R		Inositol transporter 1 (INT1)
AT5G57110	372 R		313 R	Autoinhibited Ca ²⁺ -ATPase, isoform 8 (ACA8)
AT5G03140	357 F		183 F	Lectin protein kinase family protein
AT4G36930	435 R		62 F	SPATULA (SPT), bHLH type transcription factor
AT5G65110	202 F		343 F	Acyl-CoA oxidase 2 (ACX2)
AT3G19350	347 R	425 R	40 F	Maternally expressed PAB c terminal (MPC)
AT3G04120	310 R	74 F		Cytosolic GADPH (C subunit)
AT5G67300	206 F, 220 R	80 R		Myb protein 44

Supplementary Table 3. A list of oligonucleotides used in this study.

Purpose	Gene	Name	Sequence	Restriction site
Plasmid constructs				
3FLAG-FHY3-3HA SPYNE-FHY3 cLUC-FHY3N	FHY3	F	CCCCAATTGATGGATATAGATCTTCGACTACATTCAG G	MfeI
		F2	GGGGGATCCATGGATATAGATCTTCGACTAC	BamHI
		R	ACGCGTCGACCGAGTGTCTAGACGCGTCCTCCTGCA	Sall
		NF	GGCCGGTACCATGGATATAGATCTTCGACTACATTC A	KpnI
		NR	GGCCGTCGACTCAATCGCCAGATCTACTGCATA	Sall
FHY3::FHY3-LUC	LUC	F	TGGATCCATGGAAGACGCCAAAAACATA	BamHI
		R	TGAGCTCTTACAATTTGGACTTTCGCC	SacI
SPYNE-FAR1	FAR1	F	GGGGGATCCATGAGGCAGCGTATATCTTTTTATC	BamHI
		R	CCGCTCGAGTAGCTGCCTTGATGAACTACC	XhoI
3FLAG-CCA1-3HA SPYNE-CCA1 nLUC-CCA1N AD-CCA1 pGEX-4T-CCA1	CCA1	F	CGGAATTCATGGAGACAAATTCGCTGGAG	EcoRI
		R	GCGTCGACTGTGGAAGCTTGAGTTCCAA	Sall
		F2	GGGGGATCCATGGAGACAAATTCGCTGG	BamHI
		NR	GGGCTCGAGACCCTGAACTAAGAGGAGCACA	XhoI
SPYNE-LHY	LHY	F	GGGGGATCCATGGATACTAATACATCTGG	BamHI
		R	GGGGTCGACTGTAGAAGCTTCTCCTTCCAATC	Sall
pGEX-4T-LHY AD-LHY	LHY	F2	GGGCAATTGATGGATACTAATACATCTGGAG	MfeI
		F	CGGAATTCATGGATACTAATACATCTGG	EcoRI
		R	CTCGAGTCATGTAGAAGCTTCTCCTTCC	XhoI
SPYNE-HY5 AD-HY5 pGEX-4T-HY5	HY5	F	GGGGGATCCATGCAGGAACAAGCGACTAG	BamHI
		F	GGGGAATTCATGCAGGAACAAGCGACTAG	EcoRI
		R	GGGGTCGACAAGGCTTGCATCAGCATTAG	Sall
pSAT6-EYFP-ELF4	ELF4	F	AGATCTATGAAGAGGAACGGCGAG	BglII
		R	GTCGACAGCTCTAGTTCGGGCAGC	Sall
	3FLAG	F	GGAAGATCTATACCGTCGAGATGGACTACAAAGACG ATGACGATAAAGTCGAGATGGACTACAAAGACGAT GACGATAAAGTCGAGATGGACTACAAAGACGATGA CGATAAAGTCGAGGGGAATTCCG	BglII -EcoRI
		R	CGGAATTCGCCCTCGACTTATCGTCATCGTCTTTGT AGTCCATCTCGACTTTATCGTCATCGTCTTTGTAGTC CATCTCGACTTTATCGTCATCGTCTTTGTAGTCCATC TCGACGGTA TAGATCTTCC	EcoRI -BglII
	3HA	F	CCTGGTCGACTACCCATACGACGTTCCAGACTATGC GGGCTATCCCTATGACGTCCCGACTATGCAGGATC CTATCCATATGACGTCCAGACTACGCTGCTCAGTA AGGTACCAA	Sall -KpnI
		R	TTGGTACCTTACTGAGCAGCGTAGTCTGGGACGTCA TATGGATAGGATCCTGCATAGTCCGGGACGTCATAG	KpnI -Sall

			GGATAGCCCGCATAGTCTGGAACGTCGTATGGGTAG TCGACCAGG		
<i>ELF4::LUC</i> <i>ELF4::LacZ</i>	ELF4p	1600F	GGGCTGCAGGAATTCATGTTTCTATCTCTGCCCC	Pst -EcoRI	
		ATG-R	GGGGTACCGGATCCAATAATTTTAATTGTGTTTT	KpnI -BamHI	
		400F	GGGCTGCAGGAATTCCTGCACCGTGCAAGTCGTAC	Pst -EcoRI	
	EEm-1	mF	CAGAAGATAGGTAATATTAGGATCTGATTTCTCTCGACTTT		
		mR	AAAGTCGAGAGAAAATCAGATCCTAATAGTACCTATCTTCTG		
	EEm2, 3	mF	ATATTCACTTTACAATGGGTATCTTTAGATAGGGCTACAATAAGATCC TTATC		
		mR	GATAAGGATCTTATTGTAGCCCTATCTAAAGATACCCATTGTAAAGTG AATA		
	ACEm	mF	ATCAGATAGATTTGTCTTTGTGAGATA		
mR		TATCTCACAAAGACAAATCTATCTGAT			
EMSA assay					
EMSA	EE-1	WF	GATTCTACTCAGAAGATATTTACTATAAAATATCTGATTTCTCTCGACTT TG		
		WR	CTCTCTTTTGGATTTTCGTCAAAGTCGAGAGAAAATCAGATATTTATAGT A		
		mF	GATTCTACTCAGAAGATAGGTAATATTAGGATCTGATTTCTCTCGACTT T		
		mR	CTCTCTTTTGGACCTTCGTCAAAGTCGAGAGAAAATCAGATACCTATAGT		
	EE-2,3	WF	TTCACTTTACAATAAAATATCTTTAGATATTTCTAC		
		WR	GGATCTTATTGTAGAAAATATCTAAAGATATTTATTGT		
		mF	TTCACTTTACAATGGGTATCTTTAGATACCCCTAC		
		mR	GGATCTTATTGTAGGGGTATCTAAAGATACCCATTGT		
	ACE	WF	GATATCAGATAGATACGTCTACGTGAGATACACGCTCTTGTT		
		WR	CTTGATTAACAAGAGCGTGTATCTCACGTAGACGTATCTATC		
		mF	GATATCAGATAGATGAGTCTGAGTGAGATACACGCTCTTGTT		
		mR	CTTGATTAACAAGAGCGTGTATCTCACCTAGACCTATCTATC		
	ChIP assay				
	ChIP-PCR	CCA1	CF	TTAGGTCCATTAGAGTGTGAG	
			CR	GTGGACCCGGAATCTAGACGAC	
		CHE	CF	CTCCTGCCGACCGTGGTCCA	
CR			CCACACAGCT CACTTCTGCT AGG		
COP1		CF	CCGTACCAGCGCTTGAGAAAACG		
		CR	GGTGTGGTGG GATCCACCGT GG		
PHYB		CF	CGTAATTGATGATGCGATTTCGAC		
		CR	GGTTTATTCTCTCTAGTGGCCA		
EPR1		CF	ACGGATTAGAGAAAACAGATCAG		
		CR	GAGAGAGAGATATCTTGTGGCTC		
PAR1		CF	CCAATCTAGATGCGAGGTTGATCC		
		CR	TTACTTTGGTTGCTTCCACAG		
ChIP-QPCR	ELF4-FBS	F	ATCACTCACAGCTTCACTCACGC		

	ACE, EE-1	R	ACACCGAGGCGAGTAAGTTCTGTT
		Probe	56-FAM -ACGTCTACG -ZEN -TGAGATACACGCTCTTGT -3IABkFQ
	ELF4-Exon	F	CTCAATACTAGTTTCTCGTCGGGC
		R	TTAAGCTCTAGTTCCGGCAGCA
		Probe	56-FAM -TTTCACGGGT -ZEN -GGGAAGAACGGTCACGAT -3IABkFQ
	Actin	F	CACAATGTTTGGCGGGATTGGTGA
		R	TGTAATTCCTTTCCGGTGGAGCAA
		Probe	56-FAM -TGGCGCCGA -ZEN -GCAGTATGAAGATCAAA -3IABkFQ

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