RESEARCH ARTICLE

Transcription Factors FHY3 and FAR1 Regulate Light-induced *CIRCADIAN CLOCK ASSOCIATED1* Gene Expression in Arabidopsis

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Short title: FHY3 and FAR1 Regulate *CCA1* Expression

One-sentence summary: Members of the photosensory-signaling pathway orchestrate the expression of the circadian clock gene *CCA1* to regulate plant responses to daily changes in the light environment.

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ABSTRACT

The circadian clock provides a time-keeping mechanism that synchronizes various biological activities with the surrounding environment. *Arabidopsis thaliana CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), encoding a MYB-related transcription factor, is a key component of the core oscillator of the circadian clock, with peak expression in the morning. The molecular mechanisms regulating the light induction and rhythmic expression of *CCA1* remain elusive. In this study, we show that two phytochrome signaling proteins, FAR-RED ELONGATED HYPOCOTYL3 (FHY3)

and its paralog FAR-RED IMPAIRED RESPONSE1 (FAR1), are essential for the light-induced expression of *CCA1*. FHY3 and FAR1 directly bind to the *CCA1* promoter and activate its expression, whereas PHYTOCHROME INTERACTING FACTOR5 (PIF5) directly binds to its promoter and represses its expression. Furthermore, PIF5 and TIMING OF CAB EXPRESSION1 physically interact with FHY3 and FAR1 to repress their transcriptional activation activity on *CCA1* expression. These findings demonstrate that the photosensory-signaling pathway integrates with circadian oscillators to orchestrate clock gene expression. This mechanism might form the molecular basis of the regulation of the clock system by light in response to daily changes in the light environment, thus increasing plant fitness.

INTRODUCTION

The circadian clock generates and maintains ~24-hour rhythms that help organisms anticipate and synchronize various developmental and physiological activities with the diurnal light/dark changes in the environment, thus enhancing plant fitness (Michael et al., 2003; Dodd et al., 2005). In the model plant species *Arabidopsis thaliana,* the central oscillator of the clock is believed to be composed of a series of transcriptional feedback loops, in which two morning-expressed single MYB-related transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) directly repress the expression of evening clock genes such as *TIMING OF CAB EXPRESSION1* (*TOC1*; also known as *PSEUDO-RESPONSE REGULATOR 1/PRR1*), *EARLY FLOWERING4* (*ELF4*), *LUX ARRHYTHMO* (*LUX*, also known as *PHYTOCLOCK1*), *PRR7*, and *PRR5* by directly binding to the evening element (EE) motifs in their promoters (Alabadí et al., 2001; Hazen et al., 2005; Perales et al., 2007; Li et al., 2011; Kamioka et al., 2016). In turn, the expression of *CCA1* and *LHY* is repressed in a sequential manner by PRR9 (morning expressed), PRR7 (midday-expressed), PRR5 (afternoon-expressed), and then TOC1 (evening-expressed) from noon until about midnight (Nakamichi et al., 2010; Huang et al., 2012). In addition, other components of the clock, such as *CCA1 HIKING EXPEDITION* (*CHE*), *LUX*, *BROTHER OF LUX ARRHYTHMO* (*BOA*, also known as *NOX*), *PROTEIN ARGININE METHYL TRANSFERASE5* (*PRMT5*), *EARLY FLOWERING3* (*ELF3*), and *ELF4* also participate in the regulation of *CCA1*

(Pruneda-Paz et al., 2009; Helfer et al., 2011; Dai et al., 2011; Deng et al., 2010; Kikis et al., 2005). However, the detailed molecular mechanisms remain largely unknown.

A key feature of the clock is that it has an intrinsic ability to reset its activity to synchronize with the surrounding environment. Light is a major signal for resetting the clock through the informational "input" pathway. Cryptochromes and phytochromes, which are photoreceptors for blue/UV-A and red/far-red light, respectively, are required for transducing the light signal to the central clock (Somers et al., 1998; Yanovsky et al., 2000). *CCA1* and *LHY* expression is induced by light, allowing them to initiate and set the phase of various rhythmic activities (Kikis et al., 2005; Wang et al., 1997). Two TCP transcription factors (TCP20 and TCP22) that are directly involved in light-induced activation of *CCA1* expression at dawn have been identified recently (Wu et al., 2016). In addition, the phytochrome-interacting factor (PIF) family of transcription factors was reported to mediate the connection between photosynthate signaling and the clock by direct binding to the promoters of *CCA1* and *LHY* in a sucrose-dependent manner (Shor et al., 2017). However, there are conflicting reports on the roles of PIFs in regulating the clock (Leivar et al., 2009; Martinez-Garcia et al., 2000; Nusinow et al., 2011; Viczian et al., 2005). Moreover, whether these transcription factors are directly involved in connecting phytochrome-mediated light signaling to the clock has not been resolved. Thus, the molecular mechanisms by which light activates *CCA1* expression and resets the clock remain poorly understood.

The phytochrome signaling intermediate FHY3 plays an important role in gating red light signaling to the clock during the daytime (Allen et al., 2006). FHY3 and its paralog FAR1 are transposase-derived transcription factors that directly activate the expression of the evening gene *ELF4* (Li et al., 2011; Lin et al., 2007). In the current study, we show that FHY3 and FAR1 are also required for the light induction and normal rhythmic expression of *CCA1* by directly binding to its promoter and activating its expression. In addition, we show that their activity is antagonized by PIF5 and TOC1 through physical interactions. Our results expand our understanding of the biological roles of FHY3 and FAR1 and provide important insights into the

molecular mechanisms regulating *CCA1* activation and resetting of the clock by light signals.

RESULTS

FHY3 and FAR1 Are Required for Light-induced *CCA1* Expression

CCA1 expression is quickly induced and initiates its oscillation when dark-grown seedlings are exposed to light (Kikis et al., 2005). To identify the signaling components involved in light-induced *CCA1* expression, we examined the effects of light treatment on 5-d-old etiolated seedlings including wild type (Col), various light signaling mutants (*phyA-211*, *phyB-9*, *phyA phyB*, *phyABDE*, *fhy3-11, far1-4, fhy3-11 far1-4*, *hy5-215*, *pif1*, *pif3*, *pif4*, *pif5*, *pif4 pif5, pifq*), and transgenic line *35S:PIF5-HA*. Etiolated seedlings were given a brief light exposure (1 min of white light, WL) and returned to darkness for 2h prior to harvesting for RNA extraction. qRT-PCR showed that 1 min WL exposure was sufficient to induce *CCA1* expression in wild-type seedlings (ecotype Col-0), as well as *phyA-211*, *phyB-9*, *hy5* and *pif*-related seedlings. However, light-induced *CCA1* expression was severely compromised in the *fhy3-11* single mutant, *fhy3 far1* double mutant, *phyA phyB* double mutant, *phyABDE* quadruple mutant, and *35S:PIF5-HA* transgenic plants (Fig. 1A, Supplemental Fig. 1). These observations indicate that phytochromes (primarily *phyA* and *phyB*), *FHY3* and *FAR1* play important roles in the rapid induction of *CCA1* expression by light, whereas *PIF5* likely plays a repressive role in light-induced *CCA1* expression.

Next, we investigated whether light-induced activation of *CCA1* is regulated by the clock. *Arabidopsis* seedlings were clock entrained (grown under 12h light and 12h dark cycle for 5 days) and then released into continuous darkness to maintain *CCA1* at a steady low level. The seedlings were then exposed to white light for 1 h at various time points (ZT40, ZT44, ZT48, ZT52, ZT56, ZT60, ZT64, ZT68, and ZT72) and harvested immediately after the light treatment (at ZT41, ZT45, ZT49, ZT53, ZT57, ZT61, ZT65, ZT69, and ZT73). qRT-PCR showed that in wild-type seedlings, *CCA1* expression increased more significantly when the light treatment was given during the subjective early day (ZT49 and ZT53) vs. the subjective night (ZT61, ZT65 and ZT69)

(all $p < 0.001$) and that the induction was obviously compromised in the *fhy3 far1* mutant at some time points (Fig. 1B). These findings suggest that light-induced *CCA1* expression is also subjected to a gating effect of the clock, which is consistent with the finding that FHY3 plays an important role in gating red light input to the circadian clock during the subjective day (Allen et al., 2006).

FHY3 and FAR1 Directly Bind to the *CCA1* Promoter and Activate its Expression

We previously showed that FHY3 and FAR1 are associated with the *CCA1* promoter in vivo (Li et al., 2011), suggesting that *CCA1* might be a direct downstream target of FHY3 and FAR1. Bioinformatic analysis of the *cis*-elements in the *CCA1* promoter revealed that, besides the known TOC1 binding sites (T1ME), G-box element, ACE element, CHE binding site (TBS) and LUX binding site (LBS), there is a putative FHY3/FAR1-binding site (FBS, with the sequence CACGCGC, nucleotides -694 to -700) (Fig. 2A). Thus, we performed a yeast one-hybrid (Y1H) assay and an electrophoretic mobility shift assay (EMSA) to determine whether FHY3 and FAR1 directly bind to the *CCA1* promoter. Both assays showed that indeed FHY3 and FAR1 directly bound to the FBS element, whereas mutations in the FBS element abolished the DNA-binding activity of FHY3 and FAR1 (Fig. 2B and C), indicating that the binding is specific. Next, we performed a transient expression assay to examine the regulatory effect of FHY3 and FAR1 on *CCA1* expression in *Nicotiana benthamiana* leaf cells. Both FHY3 and FAR1 activated the expression of the *CCA1p:LUC* reporter (Fig. 2D). Furthermore, a mutation in the FBS motif in the *CCA1* promoter abolished this activation by FHY3 and FAR1. These observations suggest that FHY3 and FAR1 positively regulate *CCA1* expression by directly binding to the FBS motif in its promoter.

Next, we investigated whether this direct binding of FHY3 to the *CCA1* promoter is required for the rapid induction of *CCA1* expression by light. We generated transgenic plants expressing a luciferase (LUC) reporter gene driven by wild type (*CCA1p*) and FBS mutated forms of the *CCA1* promoter (*CCA1p-FBSm*). As expected,

the *CCA1p-LUC* reporter gene was rapidly induced by 1 min of WL treatment in the wild-type background, but not in the *fhy3* background (Fig. 2E). However, the LUC reporter gene driven by the *CCA1* promoter with mutated FBS (*CCA1p-FBSm*) lost the response to light (Fig. 2E). These observations indicate that the direct interaction between FHY3 and the *CCA1* promoter is indispensable for the induction of *CCA1* by light. Immunoblot analysis showed that the accumulation of FHY3 and FAR1 was significantly enhanced by 1 min WL treatment (Fig. 2F), although the *FHY3* transcript level was only mildly upregulated (Fig. 2G). Consistent with this finding, a chromatin immunoprecipitation (ChIP) assay showed that the enrichment of FHY3 on the *CCA1* promoter substantially increased in response to light treatment (Fig. 2H).

To further investigate the effect of FHY3 on *CCA1* induction, we treated *FHY3p:FHY3-GR fhy3-4* transgenic plants (Lin et al., 2007) (FHY3 protein fused with a dexamethasone-inducible [Dex] glucocorticoid receptor [GR]) with DMSO or Dex for 2h, exposed them to WL for 1 min, and incubated them in the dark for 2 h before tissue harvest. qRT-PCR showed that brief (1 min) exposure to WL after Dex treatment (but not DMSO treatment) induced *CCA1* expression; however, Dex treatment alone did not induce *CCA1* expression (Fig. 2I). Immunoblot assays showed that FHY3 protein levels were similar in DMSO- and Dex treated samples (Supplemental Fig. 2), indicating that both the nuclear localization of FHY3 (triggered by Dex treatment) and light treatment are required for light-induced *CCA1* expression.

PIF3 and PIF5 Directly Bind to the *CCA1* Promoter

Given the presence of a G-box element in the *CCA1* promoter and the in vivo binding of PIF proteins to the *CCA1* promoter (Shor et al., 2017), we also investigated whether the PIFs directly bind to the *CCA1* promoter. A Y1H assay showed that only PIF3 and PIF5, but not PIF1 and PIF4, specifically binds to the G-box element in the *CCA1* promoter (Fig. 3A). We further confirmed the binding of PIF3 and PIF5 to the *CCA1* promoter by EMSA (Fig. 3B). Transient expression assays showed that PIF5, but not PIF3, significantly repressed the expression of the *CCA1p:LUC* reporter in

Nicotiana benthamiana leaf cells (Fig. 3C). Interestingly, the repressive effect of PIF5 was still present even when the G-box motif in the *CCA1* promoter was mutated (Fig. 3C). This observation suggests that the repressive effect of PIF5 on *CCA1* expression is independent of its DNA binding activity.

FHY3 and PIF5 Are Required for the Normal Rhythmic Expression of *CCA1*

We then investigated the roles of FHY3, FAR1, and PIFs in regulating the rhythmic expression of *CCA1 in planta* by comparing the diurnal *CCA1* expression patterns in the wild type (No-0 and Col-0 ecotypes), *fhy3-4* single mutant, *fhy3-4 far1-2* double mutant, and several *pif*-related lines (*pif3*, *pif5*, *pif4 pif5*, *pifq*, *35S:PIF3-myc* and *35S:PIF5-HA*). The seedlings were grown in 12L:12D conditions for 7 days before being transferred to continuous light conditions. qRT-PCR revealed that under free-running conditions, the amplitude of *CCA1* expression was significantly reduced in the *fhy3-4*, *fhy3-4 far1-2*, and *35S:PIF5-HA* overexpression transgenic plants, but not in the *pif* mutants or *35S:PIF3-myc* transgenic plants (Fig. 4A and B; Supplemental Fig. 3). Similarly, *LHY* expression level was also reduced in *fhy3-11* and *35S:PIF5-HA* transgenic plants under these conditions (Supplemental Fig. 4). To confirm these observations, we introduced the *CCA1:LUC* reporter (Salomé and McClung, 2005) into the *fhy3-11*, *35S:Flag-FHY3-HA, 35S:PIF3-myc* and *35S:PIF5-HA* backgrounds and found that the activity of *CCA1:LUC* was severely reduced in the *fhy3-11* background but increased in the *35S:Flag-FHY3-HA* background under continuous light conditions, compared to the wild type (Fig. 4C). In addition, *CCA1:LUC* expression was notably reduced in the *35S:PIF5-HA* transgenic background but appeared to be only slightly reduced in the *35S:PIF3-myc* transgenic background (Fig. 4D). These observations further support our conclusion that FHY3 and FAR1 positively regulate *CCA1* expression, while PIF5 negatively regulates *CCA1* expression, under diurnal cycle conditions.

TOC1 and PIF5 Interact with FHY3

Considering the direct binding of FHY3, FAR1, PIF5 and TOC1 (this study and Li et al., 2011) to the *CCA1* promoter, we speculated that FHY3 (and probably FAR1), PIF5 (and probably PIF3) and TOC1 coordinately regulate *CCA1* expression through the formation of higher-order protein complex(es). To test this possibility, we conducted pair-wise protein-protein interaction studies using yeast two-hybrid assay. Both FHY3 and FAR1 interacted with PIF1, PIF3, PIF5 and TOC1, but not with other PRR family members (PRR9, PRR7 and PRR5) (Fig. 5A, Supplemental Fig. 5 and 6). Domain deletion analysis revealed that the C-terminal domain of PIF5 (including the bHLH motif) and the central linker domain of TOC1 are responsible for the interaction with FHY3 (Supplemental Fig. 7A and B). Conversely, the central transposase domain and C-terminal SWIM domain of FHY3 are required for the interactions with PIF5 and TOC1 (Supplemental Fig. 7C). The in vivo interaction between FHY3 with PIF5 and TOC1 was further confirmed using a bimolecular fluorescence complementation assay (BiFC) and a luciferase complementation imaging assay (LCI) (Fig. 5B and C).

To further confirm PIF5-FHY3 interaction *in planta*, we performed Co-IP using *35S:Flag-FHY3-HA*/*35S:PIF5-HA* double transgenic plants (generated by crossing *35S:Flag-FHY3-HA* and *35S:PIF5-HA* transgenic plants). Anti-Flag antibodies precipitated PIF5-HA along with Flag-FHY3-HA (Fig. 5D). To confirm the TOC1-FHY3 interaction *in vivo*, we generated *35S:FHY3-Flag* and *35S:Flag-TOC1-HA* transgenic plants. The *35S:FHY3-Flag* transgene successfully rescued the long-hypocotyl phenotype of the *fhy3-11* mutant under continuous far-red light conditions, suggesting that the FHY3-Flag fusion protein is biologically functional (Supplemental Fig. 8A). Similarly, *35S:Flag-TOC1-HA* transgenic seedlings displayed shorter hypocotyls than the wild type plants (Supplemental Fig. 8B), as the previously reported *TOC1* overexpression lines (Más et al., 2003), suggesting that the Flag-TOC1-HA fusion protein is biologically functional. We crossed *35S:FHY3-Flag* and *35S:Flag-TOC1-HA* transgenic plants to produce *35S:FHY3-Flag/35S:Flag-TOC1-HA* double transgenic plants. In a Co-IP assay using anti-HA antibodies, FHY3-Flag protein was pulled down together with

Flag-TOC1-HA protein (Fig. 5E). Together, these results support the physical interaction of FHY3 with PIF5 and TOC1 *in planta*.

TOC1 and PIF5 Repress the Transcriptional Activation Activity of FHY3

We performed a transient expression assay in *N. benthamiana* leaves to test the effects of FHY3-PIF3, FHY3-PIF5 and FHY3-TOC1 interactions on *CCA1* transcription. FHY3 effectively induced *CCA1p:LUC* reporter gene expression, whereas co-expression of PIF5 or TOC1, but not PIF3, with FHY3 led to significantly less induction of the *CCA1p:LUC* reporter gene (Fig. 6A-D, Supplemental Fig. 9), indicating that both PIF5 and TOC1, but not PIF3, suppress the transcriptional activation activity of FHY3. Notably, the repressive activity of PIF5 on the activation of *CCA1* expression by FHY3 was still observed when the G-box motif in the *CCA1* promoter was mutated (Supplemental Fig. 10), suggesting that PIF5 might repress FHY3 activity via a direct protein-protein interaction.

To further investigate the effects of FHY3-PIF5 and FHY3-TOC1 interactions on the rhythmic expression of *CCA1*, we examined the expression of *CCA1* in the double transgenic plants *35S:Flag-FHY3-HA/35S:Flag-TOC1-HA* and *35S:Flag-FHY3-HA/35S:PIF5-HA* . qRT-PCR analysis showed that the amplitude of *CCA1* expression was significantly reduced in these double transgenic plants, although the transcript levels of *FHY3, TOC1* and *PIF5* in these plants were comparable to those in their respective parental plants (Fig. 6E and 6F; Supplemental Fig. 11A-C). These observations support that notion that TOC1 and PIF5 play a suppressive role in FHY3-induced *CCA1* expression.

FHY3, PIF5, and TOC1 Coordinately Regulate *CCA1* Expression during the Diurnal Light/Dark Cycle

We previously showed that FHY3 protein levels oscillate and peak at dawn under diurnal light/dark cycle conditions (Li et al., 2011). To verify this finding, we performed an immunoblot assay using *35S:Flag-FHY3-HA* transgenic plants and found that the protein level of FHY3 was indeed diurnally regulated, with peak

accumulation detected at dawn (Fig. 7A). Consistent with the finding that the accumulation of *PIF5* mRNA is regulated by the clock (Nusinow et al., 2011), our immunoblot analysis showed that in the *35S:PIF5-HA* transgenic lines, PIF5 protein accumulated from daytime to dusk, peaked at ZT8-ZT16, and gradually declined before dawn (Fig. 7B). Similarly, TOC1 protein accumulation increased in the early evening and declined before dawn in the *35S:Flag-TOC1-HA* transgenic lines (Fig. 7C). To confirm the diurnal accumulation patterns of these proteins, we generated *PIF5p:PIF5-myc* transgenic plants (in which the *PIF5-myc* transgene was driven by its endogenous promoter). Immunoblot analysis of FHY3, PIF5 and TOC1 proteins in the *FHY3p:FHY3-YFP* (Lin et al., 2008), *PIF5p:PIF5-myc* and *TOC1p:TOC1-YFP* (Más et al., 2003) transgenic plants revealed similar accumulation patterns for these proteins to those observed in their respective overexpression lines (Supplemental Fig. 12). To further determine whether the association of FHY3, PIF5 and TOC1 with *CCA1* promoter is consistent with their accumulation patterns, we performed a time-course ChIP assay. The maximum binding of FHY3 to the *CCA1* promoter occurred at predawn (ZT22), whereas the maximum binding of PIF5 and TOC1 to the *CCA1* promoter occurred post dusk (ZT14) (Fig. 7D-F). These observations indicate that the dynamic accumulation patterns of FHY3, PIF5 and TOC1 are consistent with their DNA binding activities to the *CCA1* promoter.

Feedback Regulation of *CCA1* Expression

As the expression of several *PIFs* genes (*PIF1*, *PIF4* and *PIF5*) is regulated by the clock (Nozue et al., 2007; Nusinow et al., 2011; Yamashino et al., 2003), we examined the effects of the *cca1-1* mutation and *CCA1* overexpression (*CCA1-OX*) on *FHY3* and *PIF5* expression. Although the transcript level of *FHY3* did not show an obvious rhythmic pattern, it was obviously reduced in the *cca1-1* mutant and increased in the *CCA1-OX* line compared to the wild type plants (Supplemental Fig. 13A), suggesting that *CCA1* positively regulates *FHY3* expression. In addition, as CCA1 can physically interact with FHY3 (Li et al., 2011), we also examined the effect of CCA1 on the transcriptional activity of FHY3. Indeed, CCA1 repressed the

transcriptional activation activity of FHY3 on *CCA1* (Supplemental Fig. 13B). This observation is consistent with the finding that the constitutive expression of *CCA1* disrupts its rhythmic expression pattern (Wang and Tobin, 1998). Notably, the expression level of *PIF5* was also obviously reduced in the *cca1-1* mutant during the subjective day but markedly increased in the *CCA1-OX* background from day to night (Supplemental Fig. 13C). These observations suggest that *CCA1* expression is also subjected to feedback regulation by FHY3, PIF5 and itself.

DISCUSSION

We previously demonstrated that *Arabidopsis* FHY3 and FAR1, two signaling intermediates of the phytochrome pathway, are essential for activating the expression of the evening gene *ELF4* and that their activity is negatively regulated by CCA1 and LHY through physical interactions (Li et al., 2011). In the current study, we obtained multiple lines of evidence showing that FHY3 and FAR1 also play important roles in the light-induced activation of *CCA1* expression. First, we showed that *CCA1* expression in dark-grown seedlings is activated by a brief exposure of light (1 min) and that this induction is significantly compromised in the *fhy3* single and *fhy3 far1* double mutant backgrounds (Fig. 1A and B). Second, we showed that FHY3 and FAR1 can directly bind to the *CCA1* promoter through the FBS site (Fig. 2B and C). Third, we showed that FHY3 and FAR1 can activate *CCA1* expression in a transient expression assay (Fig. 2D). Fourth, we showed that FHY3 protein accumulation increased in the light (Fig. 2F). Consistent with this finding, a ChIP-PCR assay revealed that the in vivo binding of FHY3 to the *CCA1* promoter is stronger in the light than in the dark (Fig. 2H). These results convincingly demonstrate that FHY3 and FAR1 play positive roles in light-induced *CCA1* expression.

Moreover, we demonstrated that FHY3 and FAR1 physically interact with other light signaling intermediates (such as PIF5) and key components of the central oscillator (such as TOC1) to coordinately regulate the normal rhythmic patterns of *CCA1* and *LHY* expression. Both qRT-PCR and *CCA1:LUC* reporter assays showed that under free-running conditions, the amplitude of *CCA1* and *LHY* expression was

291 significantly reduced in *fhy3-4*, *fhy3-4 far1-2*, and *35S:PIF5-HA* overexpression
292 plants but increased in the *35S:Flag-FHY3-HA* background under continuous light
293 conditions (Fig. 4; Supplemental Fig. 4). These findings suggest that FHY3 and FAR1
294 positively regulate *CCA1* and *LHY* expression, whereas PIF5 negatively regulates
295 their expression.

296 We also showed that PIF5 and TOC1 physically interact with FHY3 and FAR1
297 and repress their transcriptional activation activity (Fig. 5 and 6). In addition, FHY3,
298 PIF5 and TOC1 proteins displayed distinct oscillation patterns under diurnal day/night
299 cycle conditions. Peak accumulation of FHY3 was detected at dawn, which resembles
300 the expression pattern of *CCA1* (Fig. 7A; Supplemental Fig. 11A). PIF5 protein
301 accumulation peaked at ZT8-ZT16 and then gradually declined before dawn (Fig. 7B;
302 Supplemental Fig. 11B). Similarly, TOC1 protein accumulated in the early evening
303 and declined at predawn (Fig. 7C; Supplemental Fig. 11C). These observations
304 collectively suggest that decreased accumulation of PIF5 and TOC1 and the
305 concomitant increase in FHY3 (and probably FAR1) accumulation at dawn are
306 required to lift the repressive activity of TOC1 and PIF5 on FHY3, thus allowing
307 FHY3/FAR1 to activate *CCA1* expression at dawn (Fig. 7G). This model is consistent
308 with the observation that PIF5 still repressed the transcriptional activation activity of
309 FHY3 on *CCA1* expression even when its binding site (the G-box) was mutated
310 (Supplemental Fig. 10).

311 It is worth noting that the current clock model in *Arabidopsis* is mainly based on
312 negative feedback loops formed by transcriptional repressors (Harmer, 2009). Two
313 sets of activator and co-activator systems were subsequently identified for the core
314 clock genes. Two midday-expressed MYB-like transcription factors REVEILLE4
315 (RVE4) and RVE8 form a complex with LNK1 (NIGHT LIGHT-INDUCIBLE AND
316 CLOCK-REGULATED1) and LNK2, and activate the expression of *TOC1*, *PRR5*
317 and the evening complex genes (Farinas and Mas 2011; Rawat et al. 2011; Hsu et al.
318 2013; Xie et al., 2014). Another study identified a complex composed of TCP20,
319 TCP22 and its co-activators LWDs (LWD1, LWD2) that targets the promoters of
320 *PRR9* and *CCA1* to activate their expression (Wang et al, 2011; Wu et al, 2016). The

difficulties in identifying positive regulators of clock genes using forward genetics approaches may lie in genetic redundancy and thus, more diversified approaches are needed to tackle this challenge. In the current study, we showed that FHY3 and FAR1 are required for light-induced *CCA1* expression (Fig. 1A). Moreover, we showed that the activation activity of FHY3 and FAR1 to confer the normal rhythmic expression pattern of *CCA1* is gated by the circadian clock (Fig. 1B) and regulated by their protein-protein interactions with PIF5 and TOC1 (Fig. 7G*)*. These findings provide valuable insight into the molecular mechanisms regulating the circadian clock in *Arabidopsis* and likely other plants as well.

Notably, previous reports indicated that multiple PIFs proteins (PIF1, PIF3, PIF4 and PIF5) associate with the *CCA1* and *LHY* promoters in vivo based on ChIP-PCR (Shor et al., 2017). However, evidence supporting the direct binding of PIF proteins to these promoters is currently lacking. Our yeast one-hybrid assay showed that only PIF3 and PIF5, but not PIF1 and PIF4, directly bind to the *CCA1* promoter through the G-box motif (Fig. 3A and B). The underlying reasons for this discrepancy remain unknown at this stage. Our finding that only PIF5, but not PIF3, represses *CCA1* expression is consistent with the earlier reports that these PIF proteins have both shared and distinct DNA binding targets, thus conferring both shared and distinct biological roles for these PIFs (Jeong and Choi, 2013; Pfeiffer et al., 2014). Furthermore, the expression of *PIF1, PIF4* and *PIF5*, but not *PIF3*, is regulated by the circadian clock (Nozue et al., 2007; Nusinow et al., 2011; Yamashino et al., 2003). Thus, exploring the different roles of PIF1, PIF3 and PIF4 in regulating *CCA1* expression and the circadian clock represents an interesting avenue for future research.

Two scenarios have been proposed for the role of TOC1 in repressing *CCA1* expression: TOC1 directly associates with the *CCA1* promoter to repress its transcription (Gendron et al, 2012) or acts indirectly through interactions with other DNA-binding factors (such as CHE) (Pruneda-Paz et al, 2009). However, these two scenarios are not mutually exclusive. Indeed, TOC1 interacts with PIF3 and PIF4 and represses the transcriptional activation activities of PIF3 and PIF4 on co-bound

downstream growth-related genes to mediate the circadian gating of diurnal and thermoresponsive growth (Soy et al, 2016; Zhu et al, 2016). Our results show that TOC1 represses *CCA1* expression by physically interacting with and suppressing the transcriptional activation activity of FHY3. Interestingly, our initial yeast two-hybrid assay showed that PIF5 also physically interacted with TCP20, a previously identified activator of *CCA1* expression (Supplemental Fig. 14), suggesting that PIF5 might also regulate (probably repress) the activity of TCP20. Future efforts to elucidate the functional relationships between FHY3 (and FAR1), PIF5, and TOC1 with the TCP-LWD1 complex should provide additional insights into the multi-layered regulation of *CCA1* expression.

We previously reported that FHY3 and FAR1 are positive regulators of *ELF4*, a key evening gene (Li et al., 2011). The finding that FHY3 and FAR1 also act as positive regulators of morning genes (*CCA1* and *LHY*) is intriguing. CCA1 and LHY directly bind to the promoters of evening genes (such as *TOC1* and *ELF4*) to repress their expression (Alabadí et al., 2001; Li et al., 2011). Thus, the regulatory relationship between *FHY3/FAR1*, *CCA1/LHY*, and *ELF4* is consistent with the previously described type 1 incoherent feedforward loop (I1-FFL) model (Alon, 2007). According to this model, the two arms of the FFL act in an opposite manner to regulate gene Z: X (in this case *FHY3* and *FAR1*) activates Z (in this case *ELF4*), but also activates Y (in this case, *CCA1* and *LHY*) to repress Z (*ELF4*) (Supplemental Fig. 15). At dawn, light promotes the accumulation of FHY3 and FAR1, which activate the expression of both morning genes and evening genes, but the expression of evening genes (*ELF4*) at dawn is repressed by the products of morning genes (CCA1 and LHY) and other regulators (such as PIF5), resulting in the repression of evening genes at dawn. In addition, we showed that the activation activity of FHY3 and FAR1 on *ELF4* and *CCA1* expression is regulated by their protein-protein interaction with HY5, CCA1 and LHY (Li et al., 2011), PIF5 and TOC1 (this study), respectively. Finally, we showed that the expression of *FHY3* and *PIF5* is also regulated by *CCA1* (Supplemental Fig. 13) and that CCA1 also represses the transcriptional activation activity of FHY3 on *CCA1* itself (Supplemental Fig. 13B). Therefore, it is apparent

that key components of the light input pathway and the central oscillator form multiple interlocking feedforward loop (FFL) circuits to generate the proper temporal expression patterns for the clock genes. Although it is a daunting task, it would be rewarding to uncover the transcriptional networks and different types of FFLs that constitute the molecular bases of the biological clock using a combination of mathematical modeling and experimental approaches.

METHODS

Plant Materials and Growth Conditions

The wild-type *Arabidopsis thaliana* plants used in this study were of the Col-0 ecotype unless otherwise indicated. The *fhy3-4*, *far1-2*, *fhy3-4 far1-2*, *FHY3p:FHY3-YFP*, *FHY3p:FHY3-GR/fhy3-4*, *35S:Flag-FHY3-HA* and *35S:Flag-FAR1-HA* plants were in the No-0 ecotype background and were described previously (Li et al., 2011; Lin et al., 2007, 2008). The *pif1-1*, *pif3-3*, *pif4-2*, *pif5-3*, *pif4 pif5* and *pifq* mutants (Leivar et al., 2008), *35S:PIF3-myc* (Feng et al., 2008), *35S:PIF5-HA* (de Lucas et al., 2008) and *TOC1p:TOC1-YFP* (Más et al., 2003) were in the Col-0 ecotype background. The *cca1-1* and *CCA1-OX* were in Wassilewskija-2 ecotype background (Green and Tobin, 1999; Wang and Tobin, 1998). *fhy3-11* (SALK_002711) and *far1-4* (SALK_031652) was obtained from the ABRC. The *CCA1:LUC* reporter line was described previously (Salomé and McClung, 2005). The *35S:Flag-TOC1-HA*, *35S:FHY3-Flag*, and *PIF5p:PIF5-myc* transgenic plants (all in the Col-0 ecotype background) were generated in this study (see below). The *35S:Flag-FHY3-HA*/*35S:Flag-TOC1-HA* and *35S:Flag-FHY3-HA*/*35S:PIF5-HA* double transgenic lines were obtained by crossing *35S:Flag-FHY3-HA* with the *35S:Flag-TOC1-HA* and *35S:PIF5-HA* line, respectively. Plants were grown on MS medium containing 2% sucrose and 0.75% agar under continuous light or 12-h light/12-h dark conditions (75 μmol $m^{-2}s^{-1}$) in a Percival growth chamber (Percival Scientific, cool white fluorescent bulb at 22℃).

Plasmid Construction

All plasmids were constructed using an In-Fusion HD cloning kit (Clontech). To generate the *CCA1p-FBS:LacZ* and *CCA1p-FBSm:LacZ* reporter constructs, oligonucleotides were synthesized as two complementary oligo primers with an *EcoRI* site overhang at the 5' end and an *XhoI* site overhang at the 3' end (see Supplemental Data Set 1). The oligo primers were annealed, and the double-stranded oligonucleotides were ligated into the *EcoRI-XhoI* sites of the *pLacZi2μ* vector (Lin et al., 2007). The *CCA1* promoter fragment (1.1 kb from ATG site) was cloned into the *pAbAi* vector (Clontech) digested with *HindIII* and *XhoI*, creating *CCA1p-AbAi*. For mutagenesis of the FBS and G-box sites in the *CCA1* promoter, primers harboring mutation sites and overlapping with the *cis*-elements were used to amplify the *CCA1* promoter fragments containing the mutated *cis*-elements. The two PCR products were used as the templates for another round of overlapping PCR to obtain the mutated full-length *CCA1* promoter. AD-FHY3, AD-FAR1, AD-PIF3, AD-PIF5, LexA-FHY3, LexA-FAR1 and various deletion constructs of LexA-FHY3 were described previously (Liu et al., 2017; Xie et al., 2017). AD-TOC1 and AD-TOC1 were generated by subcloning of the full-length *TOC1* CDS into the *pEG202* and *pB42AD* vector, respectively. Various deletions of *TOC1* and *PIF5* were PCR amplified and inserted into *pEG202* or *pB42AD* to generate various domain deletion forms of LexA-TOC1 and AD-PIF5. To obtain the wild type, FBS mutated and G-box muted *CCA1* promoter-driven luciferase construct, the amplified *CCA1p-WT*, *CCA1pFBS*m and *CCA1p-G-boxm* were individually subcloned into the *pPZP221-ELF4:LUC* vector (Li et al., 2011) through *PstI*/*BamHI* sites.

To generate *35S:FHY3-Flag* transgenic plants, the *FHY3* CDS was amplified and subcloned into *pCAMBIA1300-221-Flag* (Ren et al., 2014) through the *XbaI* site to generate the *35S:FHY3-Flag* construct. To generate *35S:Flag-TOC1-HA* transgenic plants, the full-length coding sequence of *TOC1* was digested with *EcoRI* and *SalI*. Fragments of 3×Flag, TOC1 and 3×HA were ligated together and inserted into the *pSAT6-MCS* vector (Tzfira et al., 2005) digested with *BglII* and *KpnI* to produce the *pSAT6-Flag-TOC1-HA* construct. The expression cassette of *35S:Flag-TOC1-HA* was released by *PI-PspI* digestion and inserted into the *pRCS2-OCS-Bar* vector (Tzfira et

al., 2005) to produce the *pRCS2-Flag-TOC1-HA* construct. To generate *PIF5p:PIF5-myc* transgenic plants, the genomic region of *PIF5* was amplified and inserted into the *pSPYNE-35S* vector digested with *HindIII*/*SalI* to generate the *PIF5p:PIF5-myc* construct. The *35S:FHY3-Flag*, *35S:Flag-TOC1-HA* and *PIF5p:PIF5-myc* constructs were transformed into *Arabidopsis* via agrobacteria-mediated transformation (Clough and Bent, 1998). Positive transgenic lines (at least 10 independent lines) were selected on MS medium based on kanamycin (50 mg/L) or hygromycin (50 mg/L) resistance and subjected to immunoblot analysis.

Yeast Assays

Yeast one-hybrid and yeast two-hybrid assays were performed as described previously (Liu et al., 2017).

Gene Expression Analysis

The seedling samples were harvested, frozen immediately in liquid nitrogen, and stored at -80°C until use. Two biological replicates were set up for each time point. For each replicate, 30 to 40 seedlings were harvested. The samples were ground in a grinder with a 3 mm steel ball in liquid nitrogen. Total RNA was extracted from the seedlings using Trizol (Invitrogen) following the manufacturer's protocols. The first-strand cDNA was synthesized from 1 μg of RNA using reverse transcriptase (Tiangen, FastQuant RT Kit) following digestion with gDNase from the kit to remove genomic DNA contamination. The cDNA was diluted 1:10 and subjected to quantitative PCR using SuperReal PreMix Plus (Tiangen) and a 7500 Real Time PCR System (Applied Bio-systems) cycler according to the manufacturer's instructions. The following thermal cycling profile was used: 95°C for 15 min, ~40 cycles of 95°C for 10 s and 60°C for 32 s, followed by a melt curve ranging from 65 °C to 95°C with increments of 0.5°C for 5 s. The comparative CT method was used to determine relative gene expression levels, with the expression of *PP2A* used as the internal control. Efficiency calculations assume amplicon doubling during every cycle when

measuring differences in expression. Mean values of $2^{-\Delta CT}$ ($\Delta CT = CT, gene\ of\ interest - CT, PP2A$) were calculated from three technical repeats. Primers are listed in Supplemental Data Set 1. All experiments were replicated two or three times with similar results.

Chromatin Immunoprecipitation (ChIP)

35S:Flag-FHY3-HA and *35S:PIF5-HA* transgenic seedlings were used in the ChIP assays as described previously (Liu et al., 2017). Briefly, ~2 g seedling tissue was cross-linked for 10 min in 1% formaldehyde solution under a vacuum. The cross-linked chromatin complex was isolated using nuclear lysis buffer (50 mM Tris-HCl at pH 8.0, 10 mM EDTA, and 1% SDS, PMSF and protease inhibitor cocktail), diluted five-fold in ChIP dilution buffer (16.7 mM Tris-HCl at pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, PMSF and protease inhibitor cocktail), and sheared by sonication. The sonicated chromatin complex was immunoprecipitated using anti-HA antibodies (2 μl; *Cali-Bio*). The beads were washed with low-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 150 mM NaCl and 1% Triton X-100), high-salt buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 500 mM NaCl and 1% Triton X-100), LiCl buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% NP-40 and 0.5% deoxycholate), and TE buffer (10 mM Tris-HCl at pH 8.0 and 1 mM EDTA) and eluted with elution buffer (1% SDS and 0.1 M $NaHCO_3$). After reverse cross-linking, the DNA was precipitated with phenol/chloroform/isoamyl alcohol and analyzed by ChIP-qPCR. Primers used for ChIP-qPCR are listed in Supplemental Data Set 1.

EMSA

EMSA was performed using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions. GST-FHY3N, GST-FAR1N, and GST-PIF5 bHLH fusion proteins were described previously (Liu et al., 2017; Xie et al., 2017). The oligonucleotide sequences of biotin-labeled probes are listed in

Supplemental Data Set 1. Briefly, biotin-labeled probes were incubated for 20 min with the expressed proteins in binding buffer at room temperature. The DNA-protein complexes were separated on 6% native polyacrylamide gels, and the signal was detected using the Biostep Celvin S420 system (Biostep, German).

Bimolecular Fluorescence Complementation (BiFC) Assay

The CDSs of *FHY3* and *FAR1* were amplified and cloned into the *pSPYNE-35S* vector digested with *BamHI*/*SalI* to generate FHY3-nYFP and FAR1-nYFP. The CDSs of *TOC1* and *PIF5* were subcloned into *pSPYCE-35S* to generate TOC1-cYFP and PIF5-cYFP. The nYFP and cYFP-related constructs were transformed into *A. tumefaciens* strain EHA105. Agrobacterium cultures containing the combination of nYFP and cYFP constructs were incubated for 2 h and infiltrated into 3-week-old *N. benthamiana* leaves. Reconstitution of YFP fluorescence was observed under a confocal microscope (Zeiss, LSM 700) with the following YFP filter setup: excitation at 515 nm and emission at 525 to 560 nm.

Co-IP Assay

For co-IP assays using *Arabidopsis* seedlings, total proteins were homogenized in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.1% Tween 20, 1 mM PMSF, 1× protease inhibitor cocktail) and centrifuged twice at 12,000g. The cleared extract was mixed with anti-Flag or anti-HA magnetic agarose beads (MBL) and incubated overnight at 4°C. After washing five times with coimmunoprecipitation washing buffer (100 mM NaCl and 20 mM Tris-HCl, pH7.6), the magnetic agarose beads were resuspended in extraction buffer. For immunoblot analysis, samples were separated by SDS-PAGE, and the target protein was detected using anti-Flag (1:4,000) or anti-HA (1:5,000) antibodies (MBL; M185-7 or M180-7, respectively).

LCI Assay

The firefly LCI assays were performed using *N. benthamiana* leaves. The CDSs of *FHY3* and *TOC1* were ligated into the *KpnI*/*SalI* sites of the *p1300-35S-cLUC* vector (Chen et al., 2008). For the nLUC-FHY3 and nLUC-PIF5 constructs, the CDSs of *FHY3* and *PIF5* were ligated into the *KpnI*/*SalI* sites of the *p1300-35S-cLUC* vector (Chen et al., 2008). Both the nLUC- and cLUC-fused proteins were coinfiltrated into *N. benthamiana* leaves via *Agrobacterium tumefaciens*-mediated coinfiltration. The infiltrated plants were incubated for 3d and examined using the NightSHADE LB985 Plant Imaging System (Berthold).

Transient Expression in *Nicotiana benthamiana* Leaf Cells

The *CCA1* promoter was amplified and cloned into the *pGreenII 0800-LUC* vector (Hellens et al., 2005) to generate *CCA1p:LUC*. The CDSs of *FHY3*, *PIF3*, *PIF5* and *TOC1* were amplified and subcloned into the *SPYNE* vector (Walter et al., 2004) through the *BamHI*/*SalI* sites. For transient expression, the effector and reporter constructs were coinfiltrated into *N. benthamiana* leaves via *Agrobacterium tumefaciens*-mediated coinfiltration. Luciferase and Renilla luciferase activities were quantified 3 days after transformation. To measure firefly luciferase activity, 40 μL of Lar II was added to the protein extract, and the luminescence was measured for 5 s. To measure Renilla luciferase activity, 40 μL of Stop and Glow solution was added, and the luminescence was again measured for 5 s using a Berthold LB942 luminometer.

Bioluminescence Assay

The *CCA1:LUC* reporter line (kindly provided by Rob McClung) was crossed into different mutant and transgenic backgrounds (*fhy3-11*, *35S:Flag-FHY3-HA*, *35S:PIF3-myc* and *35S:PIF5-HA*). Homozygous seedlings carrying the *CCA1:LUC* reporter were selected and used for the bioluminescence assay. Seedlings were entrained for 10d in 12-h-light/12-h-dark cycles (22°C) before being released into continuous light (22°C) conditions for LUC measurements. After spraying with 1 mM

luciferin (Goldbio), the bioluminescence generated from the *CCA1:LUC* reporter was recorded with a Top-Count luminometer.

Statistical Analysis

All statistics were calculated using SPSS software. To determine statistical significance, we employed independent *t*-test between two groups and one-way ANOVA among various genotypes. A value of $p < 0.05$ was considered to be statistically significant. All sample sizes and significance thresholds are indicated in the figure legends. The results of statistical analyses are shown in Supplemental Data Set 2.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: FHY3 (At3g22170), FAR1 (At4g15090), CCA1 (AT2G46830), PIF1 (AT2G20180), PIF3 (AT1G09530), PIF4 (AT2G43010), PIF5 (AT3G59060), TOC1 (AT5G61380).

Supplemental Data

Supplemental Figure 1. Light-induced *CCA1* Expression in *PIF*-related Mutants.

Supplemental Figure 2. Immunoblot Analysis of FHY3 Protein Levels in *FHY3p:FHY3-GR* transgenic Plants with and without Dex Treatment in both the Dark and Light.

Supplemental Figure 3. Diurnal Expression Pattern of *CCA1* in *pif3*, *pif5*, *pif4 pif5* and *pifq* Mutants.

Supplemental Figure 4. qRT-PCR Analysis Showing the Changes of *LHY* Expression in Wild-type (Col), *fhy3-11*, and *35S:PIF5-HA* Seedlings.

Supplemental Figure 5. Yeast Two-hybrid Assay Showing that PIF1, PIF3, and PIF5 Physically Interact with FHY3 and TOC1.

Supplemental Figure 6. Yeast Two-hybrid Assay Showing that FHY3 and FAR1 Interact with TOC1, but not with PRR9, PRR7, or PRR5.

588 **Supplemental Figure 7.** Mapping of the Interacting Domains of PIF5, TOC1, and
589 FHY3 Using Yeast Two-hybrid Assays.
590 **Supplemental Figure 8.** Phenotypic and Molecular Characterization of
591 *35S:FHY3-Flag* and *35S:Flag-TOC1-HA* Transgenic Plants.
592 **Supplemental Figure 9.** Transient Expression Assay in *N. benthamiana* leaves
593 Showing the Effects of Co-expressing PIF3 and FHY3 on the Expression of LUC
594 Reporter Driven by Wild Type *CCA1* Promoter.
595 **Supplemental Figure 10.** Transient Expression Assay in *N. benthamiana* Leaves
596 Showing the Effects of Co-expressing PIF5 and FHY3 on the Expression of LUC
597 Reporter Driven by *CCA1* Promoter with a Mutated G-box.
598 **Supplemental Figure 11. q**RT-PCR Analysis of the Expression Levels of *FHY3*,
599 *TOC1*, and *PIF5*.
600 **Supplemental Figure 12.** Immunoblots Showing the Oscillation Patterns of FHY3,
601 PIF5, and TOC1 under Diurnal Cycle Conditions.
602 **Supplemental Figure 13.** Feedback Regulation between *FHY3*, *PIF5*, and *CCA1*.
603 **Supplemental Figure 14.** Yeast Two-hybrid Assay Showing that PIF5 Interacts with
604 TCP20.
605 **Supplemental Figure 15.** Putative Structure of a Type 1 Incoherent Feedforward
606 Loop (I1-FFL) Composed of *FHY3/FAR1*, *CCA1/LHY*, and *ELF4*.
607 **Supplemental Data Set 1.** Primers Used in This Study.
608 **Supplemental Data Set 2.** Statistical Report of t-test and ANOVA Results for the
609 Data Presented in Each Figure.
610
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AUTHOR CONTRIBUTIONS

H.Y.W. and Y.L. designed the research and wrote the paper. H.Y.W., X.X., and P.D. supervised the work. Y.L., M.M., G.L, L.Y., Y.X., H.W., X.M., Q.L. and H.X. performed the experiments and analyzed the data.

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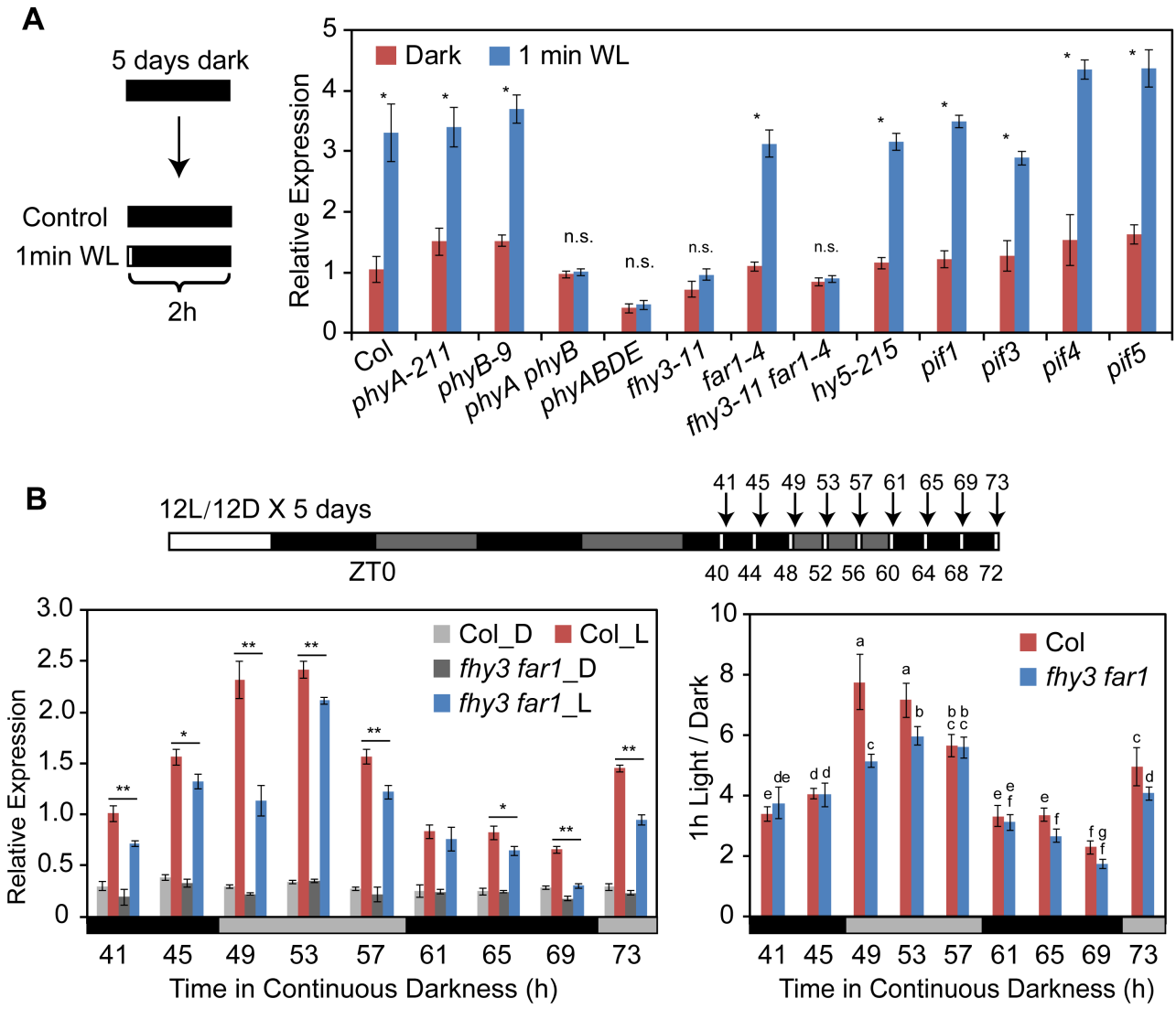

Figure 1. FHY3 and FAR1 are Required for Light-induced *CCA1* Expression. (A) qRT-PCR analysis showing the light-induced regulation of *CCA1* expression in various light signaling-related mutants. 5-d-old dark-grown *Arabidopsis* seedlings were treated with a 1 min pulse of white light and incubated in the dark for 2 h before harvesting. (*, P < 0.05, Student's *t* test, n.s. no significance). Mean ± SD (n = 3 technical replicates). Two independent biological replicates (see Methods) showed similar results. (B) qRT-PCR analysis showing the effects of light treatment at different ZTs on the expression of *CCA1*. Wild-type and *fhy3 far1* seedlings were grown in 12L:12D conditions for 5 days before being transferred to continuous darkness. Beginning at ZT44, seedlings were exposed to light for 1 h at different time points (ZT44 – ZT72) and immediately harvested for RNA extraction. Seedlings grown in the dark at the corresponding time points were used as the controls. The *CCA1* expression level was normalized to *PP2A* (*, P < 0.05, **, P < 0.01, Student's *t* test) (left panel). The ratio of *CCA1* expression in seedlings subjected to 1h light treatment versus dark-grown seedlings was used to evaluate the effects of light treatment at different time points (right panel). Different letters indicate significant differences by one-way ANOVA with SAS software (P < 0.05). Two independent biological replicates showed similar results.

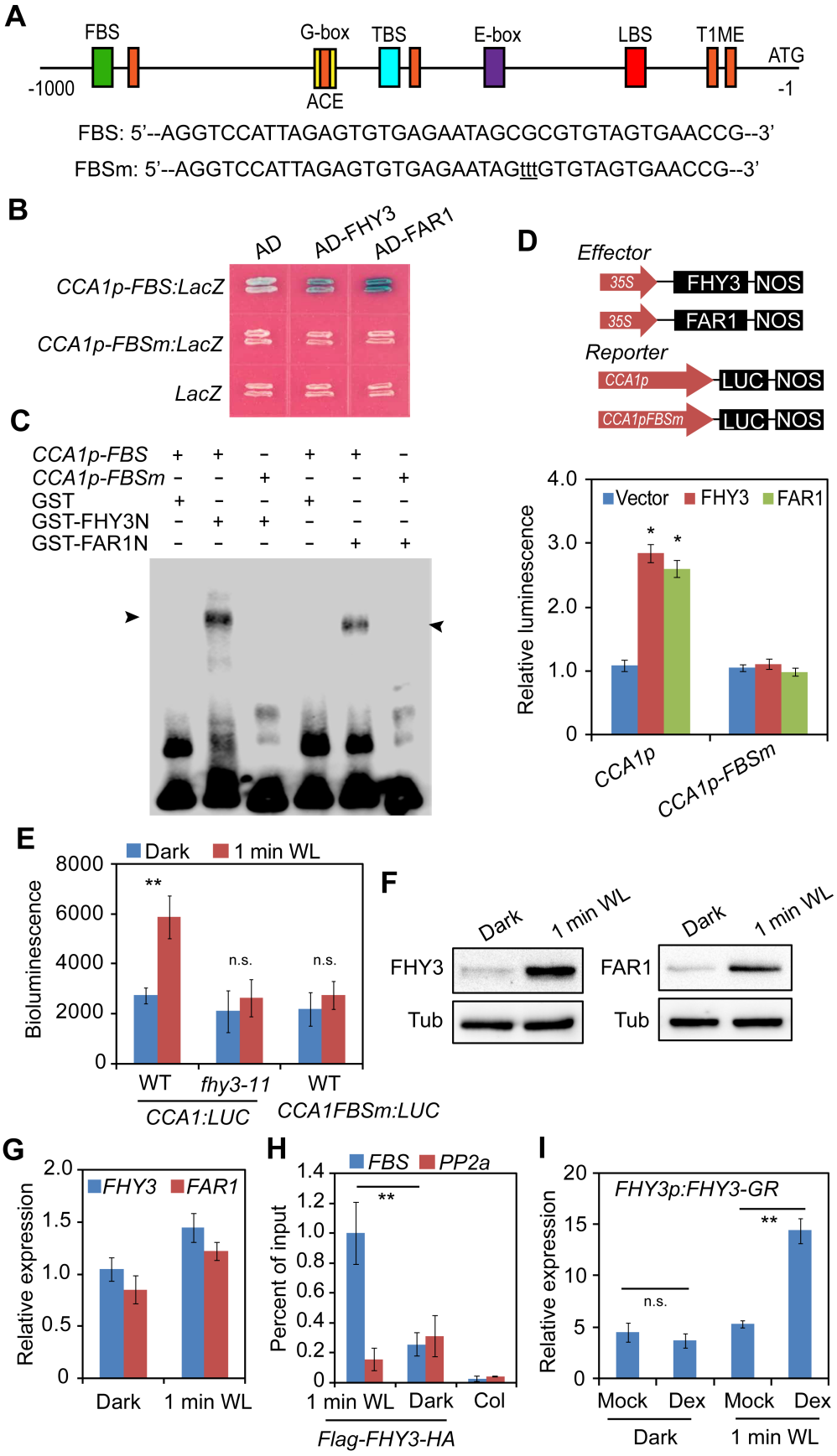
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Figure 2. FHY3 and FAR1 Directly Bind to the *CCA1* Promoter and Activate its Expression. (A) Schematic representation of the positions and nucleotide sequences of various *cis*-elements in the *CCA1* promoter. The mutations within the FBS element are shown below the diagram in lowercase letters. (B) Yeast one-hybrid assay showing that FHY3 and FAR1 directly bind to the *CCA1* promoter. The *LacZ* reporter gene was driven by the *CCA1* promoter with a wild type or mutated FBS element. Mutation of the FBS site in the *CCA1* promoter abolished the binding. (C) EMSA showing that GST-FHY3N (the first 200 amino acids of FHY3) and GST-FAR1N (the first 200 amino acids of FAR1) specifically bind to the biotin-labeled *CCA1p-FBS* probe. The arrowheads indicate GST-FHY3N and GST-FAR1N. (D) Transient expression assay showing that FHY3 and FAR1 activate *CCA1* expression in *Nicotiana benthamiana* leaf cells (*, $P < 0.05$, Student's *t* test). Mean ± SD (*n* = 3 technical replicates). Three independent biological replicates showed similar results. (E) Bioluminescence assays showing the activities of *CCA1* promoters with a wild type or mutated FBS motif in wild type or *fhy3-11* seedlings (**, $P < 0.01$, Student's *t* test, n.s. no significance). Mean ± SD (n = 3 technical replicates). Three independent biological replicates showed similar results. (F) Immunoblot assay showing increased accumulation of FHY3 and FAR1 protein in seedlings treated with 1 min WL. 5-d-old dark-grown or WL-treated *35S:Flag-FHY3-HA* and *35S:Flag-FAR1-HA* transgenic seedlings were collected for immunoblot analysis. Anti-Flag antibodies were used to detect the FHY3 or FAR1 protein. Tubulin (Tub) was used as an internal control. (G) qRT-PCR analysis showing the expression levels of *FHY3* and *FAR1* in dark-grown or 1 min WL-treated seedlings. Mean ± SD (*n* = 3 technical replicates). Two independent biological replicates showed similar results. (H) ChIP-qPCR assay showing a significant enrichment of FHY3 on the *CCA1* promoter by 1 min WL exposure. PP2A amplicon was used as a negative control. ($P < 0.01$, Student's *t* test). An independent biological replicate showed similar results. (I) qRT-PCR analysis of *CCA1* expression in *FHY3p:FHY3-GR* transgenic seedlings. 5-d-old dark-grown seedlings were treated with 20 μM Dex or DMSO (Mock) for 2h before being exposed to 1 min WL. ($P < 0.01$, Student's *t* test, n.s. no significance). Mean ± SD (n = 3 technical replicates). Three independent biological replicates showed similar results.

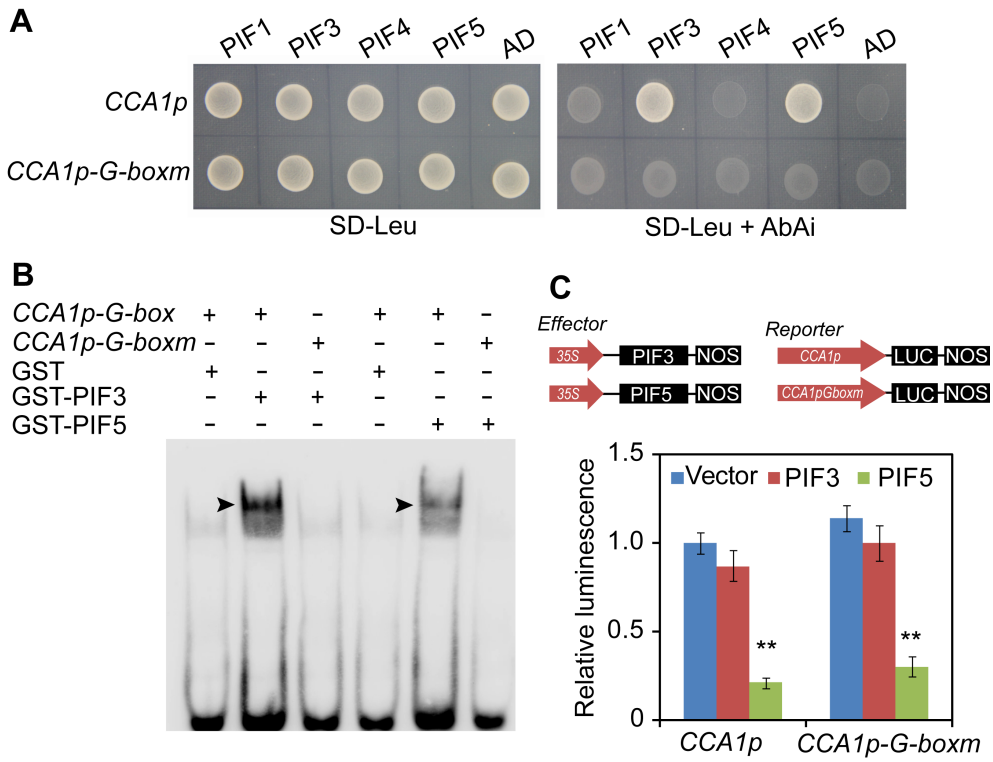

Figure 3. PIF5 Directly Binds to the *CCA1* Promoter and Represses its Expression. (A) Yeast one-hybrid assay showing that PIF3 and PIF5 directly bind to the *CCA1* promoter. PIF3 and PIF5, but not PIF1 and PIF4, activated the *AbA*^{r} reporter gene driven by the wild-type *CCA1* promoter, but not the *AbA*^{r} reporter gene driven by the *CCA1* promoter with a mutated G-box element. Empty vector expressing the AD alone was used as the negative control. (B) EMSA showing that GST-PIF3 and GST-PIF5 (bHLH, DNA-binding domain) specifically bind to the biotin-labeled *CCA1p-G-box* (right) probe. The arrowheads indicate GST-PIF3 and GST-PIF5 bHLH proteins. (C) Transient expression assay showing that PIF5 represses *CCA1* expression in *Nicotiana benthamiana* leaf cells ($P < 0.05$, Student's *t* test). Mean ± SD ($n = 3$ technical replicates). Three independent biological replicates showed similar results.

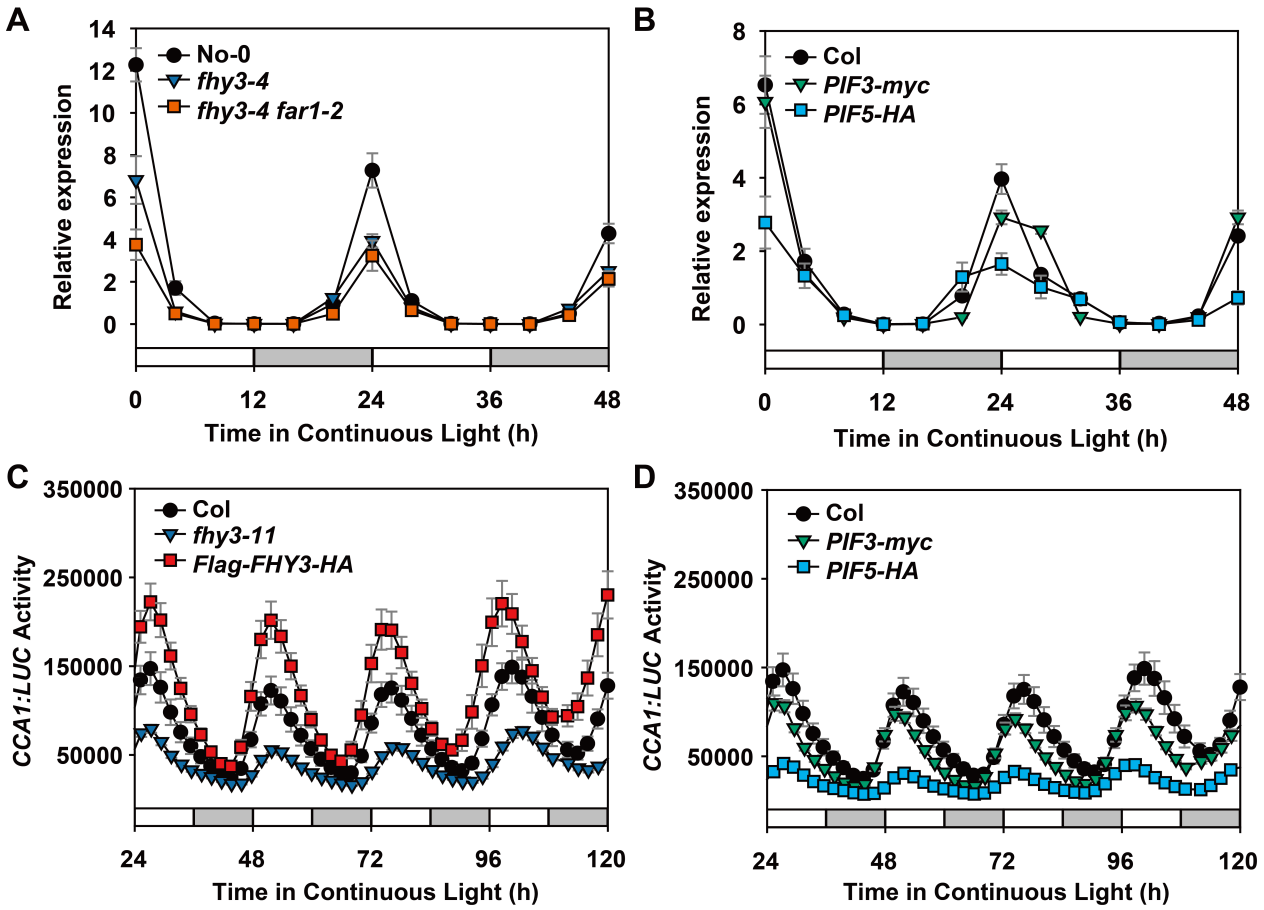

Figure 4. FHY3 and FAR1 Activate and PIF5 Represses the Rhythmic Expression of *CCA1*. (A) and (B) qRT-PCR analysis showing changes in the cyclic expression of *CCA1* in *fhy3-4* and *fhy3-4 far1-2* (A) and *35S:PIF3-myc* and *35S:PIF5-HA* plants (B). Seedlings were entrained at 22°C in 12L:12D conditions for 7 d before being released to continuous light conditions. Mean ± SD (n = 3 technical replicates). Two independent experiments were performed, with similar results. (C) and (D) Bioluminescence assays showing expression of the *CCA1:LUC* reporter in WT, *fhy3-11*, *35S:Flag-FHY3-HA*, *35S:PIF3-myc*, and *35S:PIF5-HA* plants. Seedlings carrying the *CCA1:LUC* luciferase reporter were grown under 12L:12D conditions for 7 d before being transferred to continuous white light. Mean ± SD (*n* = 3 technical replicates). Two independent biological replicates showed similar results.

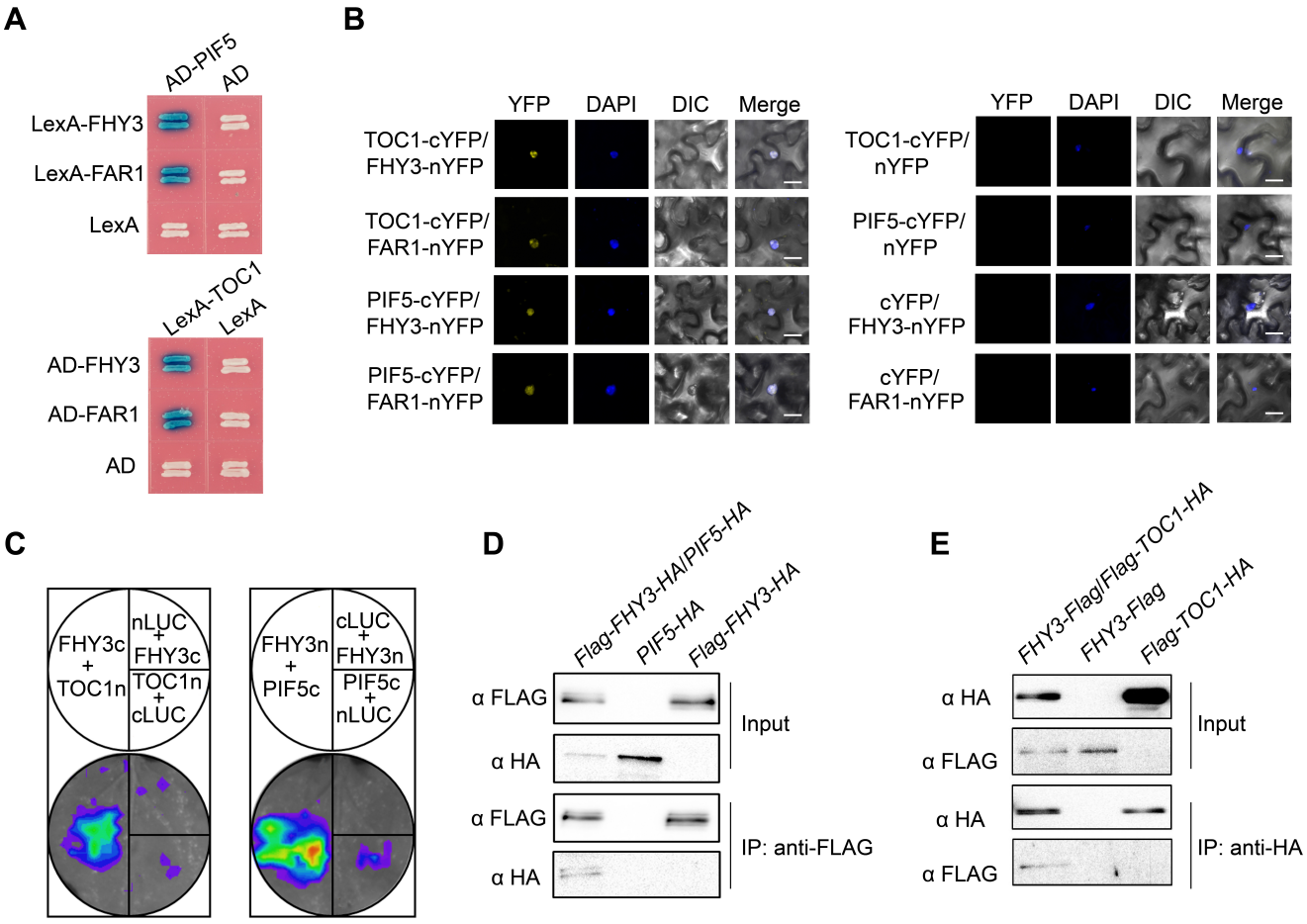

Figure 5. FHY3 Interacts with TOC1 and PIF5. (A) Yeast two-hybrid assay showing that FHY3 and FAR1 interact with PIF5 and TOC1. (B) BiFC assay showing interactions between FHY3 and TOC1, FAR1 and TOC1, FHY3 and PIF5, and FAR1 and PIF5 in *N. benthamiana* leaf epidermal cells (bars = 20 μm). FHY3 and FAR1 were fused to the N-terminal fragment of YFP (nYFP); TOC1 and PIF5 were fused to the C-terminal fragment of YFP (cYFP). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (C) Luciferase complementation imaging assay (LCI) showing that FHY3 interacts with TOC1 and PIF5 *in planta*. The C-terminal half of firefly LUC (cLUC) was fused to FHY3 or PIF5 and the N-terminal half of firefly LUC (nLUC) was fused to FHY3 or TOC1. (D) and (E) Coimmunoprecipitation assay showing that FHY3 associates with PIF5 and TOC1 *in planta*. (D) Protein extracts from seedlings expressing *35S:Flag-FHY3-HA*/*35S:PIF5-HA*, *35S:Flag-FHY3-HA*, or *35S:PIF5-HA* were immunoprecipitated with anti-Flag antibodies and detected by immunoblot analysis using anti-Flag or anti-HA antibodies. (E) Proteins extracts from seedlings expressing *35S:FHY3-Flag*/*35S:Flag-TOC1-HA*, *35S:FHY3-Flag*, or *35S:Flag-TOC1-HA* were immunoprecipitated with anti-HA antibodies and detected by immunoblot analysis using anti-HA or anti-Flag antibodies.

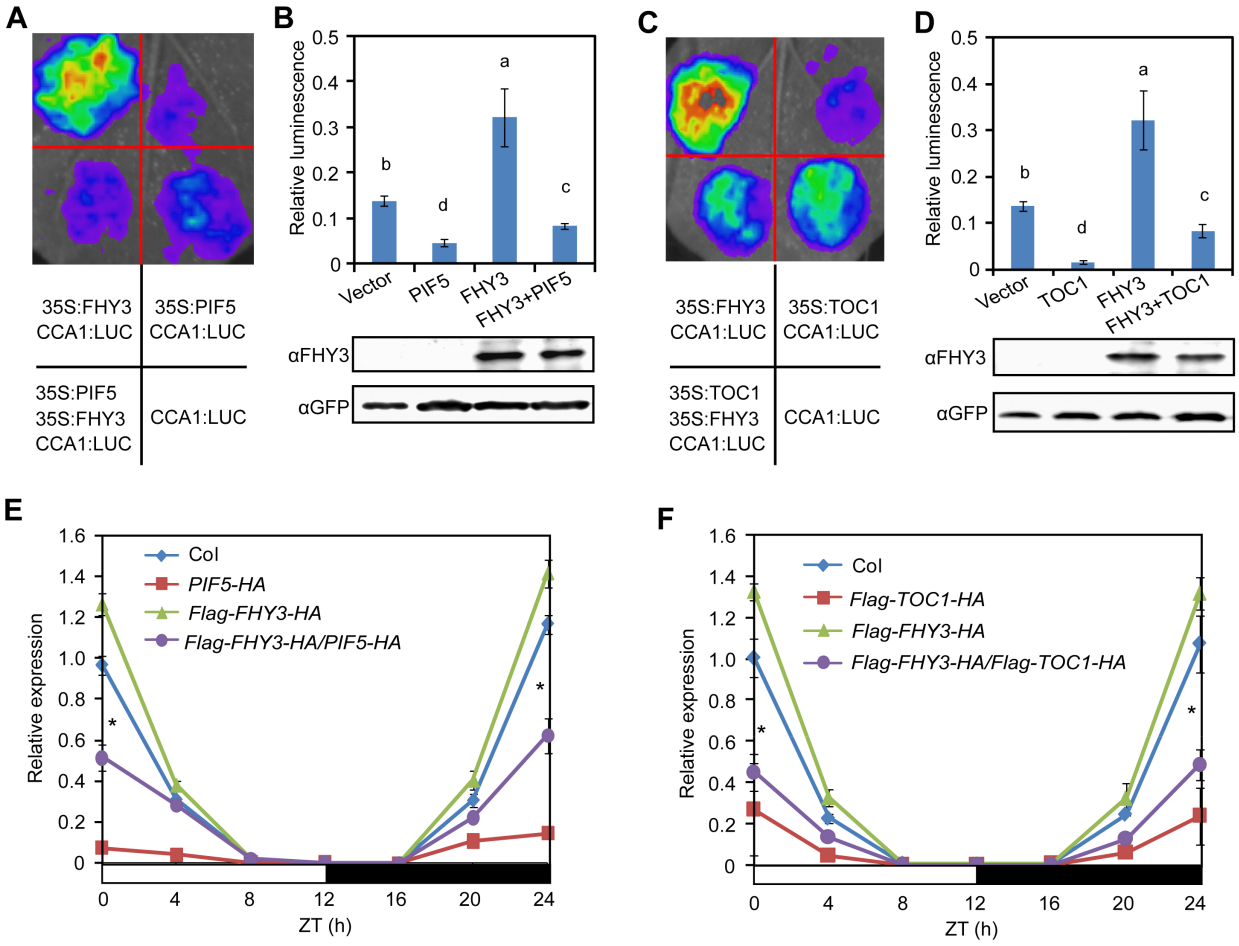

Figure 6. TOC1 and PIF5 Repress the Transcriptional Activation Activity of FHY3 on *CCA1* Expression. (A) and (B) PIF5 suppresses the activation activity of FHY3 on *CCA1* expression in *N. benthamiana* leaves. Relative LUC activity was normalized to REN activity (LUC/REN). Different letters indicate significant differences by one-way ANOVA with SAS software ($P < 0.05$). Values are mean ± SD (n = 3 technical replicates). Three independent biological replicates showed similar results. (C) and (D) TOC1 suppresses the activation activity of FHY3 on *CCA1* expression in *N. benthamiana* leaves. Different letters indicate significant differences by one-way ANOVA with SAS software ($P < 0.05$). Values are mean ± SD (n = 3 technical replicates). Three independent biological replicates showed similar results. (E) *CCA1* expression is reduced in *35S:Flag-FHY3-HA*/*35S:PIF5-HA* seedlings compared to its parental line *35S:Flag-FHY3-HA*. (F) *CCA1* expression is reduced in *35S:Flag-FHY3-HA*/*35S:Flag-TOC1-HA* seedlings compared to its parental line *35S:Flag-FHY3-HA*. For (E) and (F), seedlings were grown at 22°C in 12L:12D conditions for 7 d before being harvested for RNA extraction. (*, $P < 0.05$, Student's t test). Values are mean ± SD (n = 3 technical replicates). Two independent biological replicates showed similar results.
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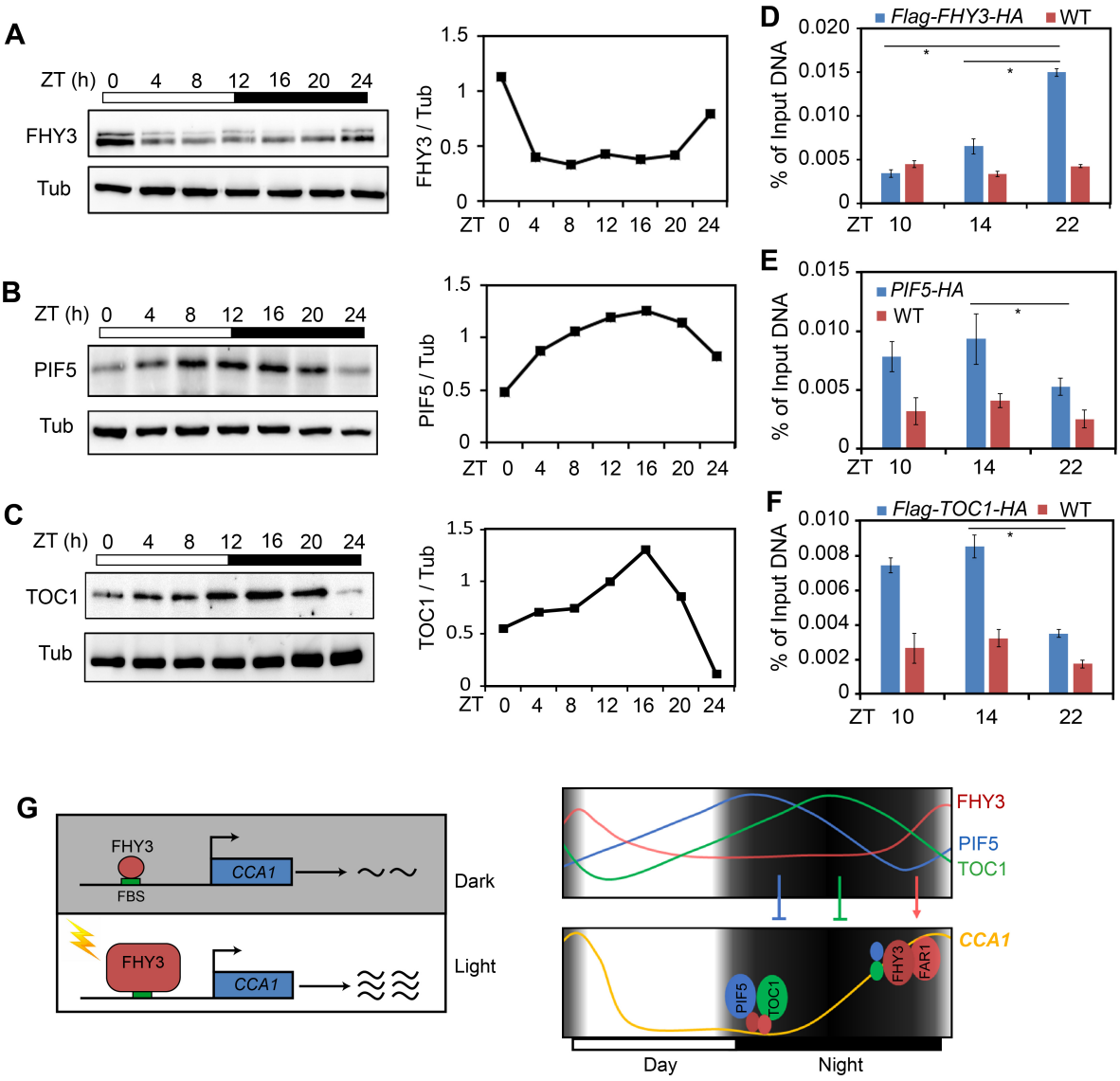

Figure 7. FHY3-TOC1 and FHY3-PIF5 Interactions Coordinately Regulate *CCA1* Expression. (A)-(C) Left panel: Immunoblot assay showing the oscillation of FHY3 (A), PIF5 (B) and TOC1 (C) protein levels under diurnal cycle conditions. Tubulin (Tub) was used as an internal control. 5-d-old, 12L:12D entrained *35S:Flag-FHY3-HA*, *35S:PIF5-HA* and *35S:Flag-TOC1-HA* seedlings were harvested at the indicated time points. Anti-Flag antibodies (1:4000; MBL) were used to detect FHY3 and TOC1. Anti-HA antibodies (1:5000; MBL) were used to detect PIF5. Right panel: Estimates of FHY3, PIF5 and TOC1 protein levels using ImageJ software based on the immunoblot results. (D)-(F) ChIP-qPCR assay showing that FHY3, PIF5 and TOC1 associate with the *CCA1* promoter at ZT10, ZT14 and ZT22. Chromatin was extracted from *35S:Flag-FHY3-HA* (D), *35S:PIF5-HA* (E) and *35S:Flag-FHY3-HA* (F) seedlings and precipitated using anti-HA antibodies. NoAb (no-antibody) precipitates and PP2a served as the negative controls. Asterisks indicate a significant difference between the indicated means with $P<0.05$ by Student's t test. Values are mean ± SD (n = 3 technical replicates). Two independent biological replicates showed similar results. (G) Schematic diagram illustrating how FHY3 mediates the light-induced expression of *CCA1* and that the role of FHY3/FAR1 in activating *CCA1* expression is antagonistically regulated by TOC1 and PIF5. After light exposure, the accumulation of FHY3 increases and it binds to the *CCA1* promoter to activate its transcription. Under diurnal cycles, TOC1 and PIF5 accumulate from day to midnight, and they repress the activity of FHY3 and FAR1 before midnight. At predawn, the protein levels of PIF5 and TOC1 decrease, thus lifting their repression of FHY3, leading to increased *CCA1* expression at dawn.