A Circadian Morning Complex Modulates Far-Red Light Signaling in *Arabidopsis*

Chen Su[1,2], Yumei Qin[1,2], Yingjun Yu[1,2], Yan Wang[1,2], Yuqing He[1,2], Seth J. Davis[3,4*], Lei Wang[1,2,5,6*]

[1] Key laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing, 10093, China

[2] University of Chinese Academy of Sciences

[3] University of York, Department of Biology, Heslington, York YO10 5DD, UK

[4] State Key Laboratory of Crop Stress Biology, School of Life Sciences, Henan University, Kaifeng 475004, China

[5] China National Botanical Garden, Beijing 100093, China

[6] Academician Workstation of Agricultural High-Tech Industrial Area of the Yellow River Delta, National Center of Technology Innovation for Comprehensive Utilization of Saline-Alkali Land, Dongying 257300, China

*Corresponding author: seth.davis@york.ac.uk and wanglei@ibcas.ac.cn

Running title: Clock Morning Complex feedback regulates far-red light signaling

Abstract

The plant circadian clock coordinates internal processes with daily and seasonal environmental changes by interacting with prevalent light cues. However, how the circadian clock feedback regulates light signals remain largely elusive. Here, we identify that the clock regulator TIME FOR COFFEE (TIC), which interacts with the core clock components CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) to form a “Morning Complex” in the nucleus and co-repress numerous genes, particularly at dawn. Intriguingly, the MC inhibits *PHYA* and other phyA signaling components at dawn through binding to their promoters. Mutants lacking CCA1 and LHY show increased sensitivity to far-red light, similar to *tic* mutants, highlighting the cooperative role of TIC, CCA1, and LHY in regulating light-inhibited hypocotyl growth. Altogether, these findings indicate that the circadian MC is a key complex, feedback regulates light signal and mediates multiple biological processes at dawn to ensure plant fitness.

Keywords: circadian morning complex, transcriptional inhibition, far-red signaling, hypocotyl growth

Introduction

Plants face significant challenges during growth and development as they cannot move to escape environmental changes, which requires them to evolve more complicated mechanisms to adapt. Light is one of the predominant external stimuli for plant growth, while the circadian clock acting as an endogenous timekeeper has multiple crosstalk with it [1-4]. There are multiple interplays between the clock and far-red (FR) light signaling [5]. phytochrome A (phyA), one of five phytochromes, mediates the perception of FR light input to the circadian clock under FR/dark cycles and alters clock periodicity [6,7]. In turn, both the transcript and protein abundance of phyA are repressed during the day, accumulating during the night, with transcript peaks late at night and protein peaks just before dawn [8-10]. Furthermore, the clock regulator TIME FOR COFFEE (TIC) acts as a negative factor for phyA to regulates *PHYA* transcription, phyA protein stability, and photobody formation, thus mediating hypocotyl growth [11,12]. Therefore, the feedback regulations are crucial for plants to synchronize growth.

TIC is a clock regulator, peaking in activity before dawn [11,13]. Its mutation shows a significantly shortened clock period and causes expression defects in morning genes, including *LHY* [13]. In addition to its role in regulating the circadian clock, TIC also plays roles in development and stress responses [14-16]. For example, we have reported that nuclear localized TIC interacts with the co-repressor TOPLESS (TPL) to inhibit the expression of a subset of genes, including *PHYA* and other far-red light signaling components like *FHY1* and *FHL* [12,13]. The transcription factors recruited by TIC to participate in this process are still unknown, and the mechanism by which TIC regulates other far-red light signaling components remains unclear.

In *Arabidopsis*, two dawn-expressed Myb-like transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), are reported as morning components of the circadian clock [17,18]. CCA1 and LHY, which colocalize in the nucleus, can form homodimers and heterodimers [19,20]. They bind a promoter element called the evening element (EE) to repress genes expression, including *PSEUDO-RESPONSE REGULATOR* (*PRR*), *GIGANTEA* (*GI*) and the

members of the evening complex (EC) [21-24]. *cca1 lhy* seedlings display shorter hypocotyl regardless of photoperiod, possibly due to the alternation of *PIF4* and *PIF5* expression patterns [25]. In addition, the expression of *CCA1* and *LHY* can be induced by light to initiate the rhythmic pace [19,26,27]. Recently, FAR-RED ELONGATED HYPOCOTYL3 (FHY3) and its paralog FAR-RED IMPAIRED RESPONSE1 (FAR1) have been found to be essential for activating *CCA1* expression through binding to its promoter [19,28]. Conversely, CCA1 inhibits the transcriptional activity of FHY3 on *FHY1* and disrupts the downstream far-red signaling pathway, resulting in the shortened hypocotyl phenotype of *cca1* mutants in constant far-red light [29].

While CCA1/LHY function as parts of a larger protein complex in plants, other components have remained unidentified [19]. For example, DE-ETIOLATED 1 (DET1), a transcriptional co-repressor, can interact with CCA1/LHY to bind and repress CCA1/LHY target genes at dawn [30]. Nevertheless, the *det1-1* mutant could not restore proper circadian oscillation of *TOC1* and *GI*, which were suppressed by CCA1-OX. This has been interpreted that other not yet identified co-repressors contribute to the transcriptional repression activities of CCA1/LHY [30]. Additionally, the *cca1 tic* and *lhy tic* double mutants exhibit shorter circadian periods under both diurnal and constant light conditions, similar to the *tic* mutants [13]. Hence, there are genetic interactions between TIC and CCA1/LHY in clock regulation.

In this study, we found that TIC interacts with CCA1 and LHY, forming a "morning complex" that coordinately co-represses genes involved in clock regulation, growth, and stress responses. This complex specifically inhibits *PHYA* and its signaling components at dawn through CCA1/LHY directly binding to their promoters. This works to regulate hypocotyl growth under far-red light. Our results reveal a key complex in which the clock feedback regulates light signals, enhancing plant fitness by matching adaptive growth responses with the optimal phototransduction timing.

Results

TIC physically interacts with CCA1 and LHY

Our previous study found TIC negatively regulates *PHYA* at the transcriptional level, but the transcription factors recruited by TIC involved in this process were not reported [12]. Given that CCA1/LHY are the morning-phased clock components that genetically interact with TIC [13], we explored whether they are the key factors recruited by TIC. First, we tested for physical interactions between TIC and CCA1/LHY proteins. Using transient co-expression of GFP-TIC and CCA1-HA or LHY-HA in *N. benthamiana* leaves, we detected the co-immunoprecipitation of CCA1-HA or LHY-HA with GFP-TIC (Fig. 1a). Next, we performed bimolecular fluorescence complementation assays (BiFC) in *N. benthamiana* leaves to examine the subcellular localizations and interactions between TIC and CCA1/LHY. As expected, we observed strong nuclear yellow fluorescence protein signals in nuclear in the presence of TIC-nYFP and CCA1-cYFP or LHY-cYFP (Fig. 1b). Next, we used the split-luciferase (SLC) imaging analysis confirmed the interaction between TIC and CCA1/LHY in tobacco leaves. (Fig. 1c). Hence, we validated the interaction between TIC and CCA1/LHY in *Arabidopsis*. The use of CCA1 endogenous antibody enables the detection of weak band in the IP from *TICpro:GFP-TIC* seedlings, while the HA antibody also allowed band detection in IP from *TICpro:GFP-TIC/LHYpro:LHY-HA hybrid seedlings*(Supplementary Fig. 1). These results indicate that TIC interacts with CCA1 and LHY under native condition. To further define the domains of TIC mediating the interaction. we conducted co-immunoprecipitation assays by co-expressing GFP-TIC-N or GFP-TIC-C with CCA1-HA or LHY-HA in *N. benthamiana* leaves. The results showed a strong interaction between GFP-TIC-N and CCA1-HA or LHY-HA (Fig. 1d). Slightly different from the co-IP results, BiFC and SLC assays showed that both TIC-NT and TIC-CT could interact with CCA1/LHY, with a stronger interaction observed for TIC-NT in the BiFC assay (Supplementary Fig. 2). Taken together, these results indicate that TIC seems to preferentially interact with CCA1 and LHY in the nucleus through its N-terminus, distinct from its

interaction domain with phyA.

TIC functions with CCA1 and LHY at dawn

To further investigate whether TIC and CCA1/LHY function together, we analyzed previously reported datasets. By overlapping ChIP-seq data of CCA1 or LHY with RNA data of *cca1 lhy* [22,31], we obtained 104 CCA1-suppressed genes, 10 CCA1-activated genes, 147 LHY-suppressed genes, and 9 LHY-activated genes (Supplementary Fig. 3). These results indicated that CCA1/LHY in *Arabidopsis* primarily act as transcriptional inhibitors in the morning. We compared our up-DEGs of *tic-2* at pre-dawn with CCA1 and LHY directly repressed targets, and found that over 61% (64/104) of CCA1-repressed genes and 55% (81/147) of LHY-repressed genes were markedly increased in *tic-2* at pre-dawn [12]. Moreover, 44 genes were co-inhibited by CCA1/LHY and TIC, indicating that CCA1 and LHY synergistically regulate a large set of genes with TIC, while also having distinct functions (Fig. 2a). To support the specificity within this regulation, we compared RNA-seq data of *tic-2* with MYC2 direct binding targets [32,33]; MYC2 is a transcription factor that can also interact with TIC [16]. These Venn diagrams showed that only 15.5% (13/84) or 7.1% (6/84) of MYC2 direct repressed genes were induced in *tic-2* at pre-dawn or post-dusk, and 5.2% (12/231) or 4.8% (11/231) of MYC2 direct induced genes decreased in *tic-2* at pre-dawn or post-dusk (Supplementary Fig. 4). This provided support that CCA1 and LHY, rather than MYC2, are the primary transcription factors recruited by TIC to form a functional complex.

Gene ontology (GO) analysis of these 44 co-repressed genes demonstrated that the terms “circadian rhythm” and “rhythmic process” were significantly enriched (Fig. 2b). These are consistent with previous findings indicating genetic interactions between TIC and CCA1/LHY in regulating clock [13]. Additionally, the biological processes “response to temperature stimulus”, “response to light stimulus”, and “response to stress” were also enriched (Fig. 2b), supporting that TIC and CCA1/LHY are coordinately involved in regulating light signaling and stress response. Heat map analysis specifically revealed the degree to which 44 co-inhibited genes increase in

both *cca1 lhy* and *tic-2*, including clock components *PRR5*, *PRR7*, *EARLY FLOWERING 4* (*ELF4*), *GI*, and others [2], growth regulator *CCG-BINDING PROTEIN 1* (*CBP1*) [34], *COLD REGULATED GENE 27* (*COR27*) [35], *COR28* [36], and others, photoreceptor *FLAVIN-BINDING, KELCH REPEAT, F BOX 1* (*FKF1*) [37], stress regulators *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2* (*DREB2A*) [38], *DREB2B* [39], and others (Fig. 2c). Next, we confirmed the expression changes of several genes in the *cca1-1 lhy-20* and *tic-2* mutants using quantitative reverse transcription PCR (qRT-PCR). We found that the transcript levels of *PRR5*, *PRR7*, *CBP1*, *DREB2A*, *DREB2B*, and *FKF1* at ZT0 were consistent with those in Fig. 2 C (Fig. 2d), whereas the changes of these genes expression at ZT12 in the *cca1-1lhy-20* and *tic-2* mutants were not concordant (Supplementary Fig. 5), further implying that the three form a complex mainly at dawn to co-suppress targets expression.

The morning complex inhibits *PHYA* and its signaling components through binding to their promoters

To understand the physiological role of CCA1 and LHY in TIC regulating *phyA* negatively, we measured the hypocotyl growth response of *cca1-1*, *lhy-20*, *cca1-1 lhy-20* mutants under constant far-red light or constant dark conditions. The *cca1-1*, *lhy-20* single or double mutants showed slightly shortened hypocotyls under a range of continuous far-red (FRc), but not in continuous darkness (Fig. 3a, b and Supplementary Fig. 6a, b), similar to *tic* mutants. We also generated their over-expressing lines in Col-0 background to test the hypocotyl phenotypes. And CCA1-OE and LHY-OE transgenic seedlings displayed longer hypocotyls under a range of continuous far-red (FRc), but not in continuous darkness (Fig. 3c, d and Supplementary Fig. 6c, d), which is consistent with previous investigation [29]. Taken together, CCA1 and LHY positively regulates far-red light-mediated hypocotyl growth. Meanwhile, we examined the expression of *PHYA* in *cca1-1*, *lhy-20* single or double mutants, and found that transcript levels of *PHYA* in the double mutant were significantly increased compared to the single mutants (Fig. 4a), suggesting functional

redundancy in transcriptional inhibition of *PHYA* by CCA1 and LHY. Moreover, the expression of *PHYA* was significantly induced in mutants at pre-dawn, not post-dusk (Fig. 4a), which is also what is seen in *tic* mutants [12], implying that the morning complex, consisting of TIC, CCA1 and LHY, functions together in repressing *PHYA*, thus regulating hypocotyl growth under FRc.

To further substantiate our hypothesis that TIC function in FR signaling necessitates CCA1 and LHY action, we further analyzed the S4, S5, and S6 regions of the *PHYA* promoter, with which TIC associates [12], identifying an EE-like element (AAAATATC) or a MYB element (ATATCT) in the S4 or S5 region of the *PHYA* promoter, directly bound by CCA1 and LHY (Fig. 4b, upper panel). Electrophoretic mobility shift assay (EMSA) showed that both purified GST-tagged CCA1 and LHY proteins, but not the GST alone, bound to the EE-like element in vitro within the S4 region of the *PHYA* promoter, and GST-CCA1 also can bind to the MYB element in the S5 region (Fig. 4b, lower panel). Unlabeled probes could compete with the binding efficiently (Fig. 4b, lower panel), supporting that CCA1 and LHY directly bind to the promoter of *PHYA*. Chromatin immunoprecipitation (ChIP)-qPCR assays using GFP-CCA1/LHY transgenic materials at pre-dawn verified these results. And the results, normalized using *APX3* as a control, demonstrated that the amplicons containing EE-like element or MYB element within *PHYA* promoter could be prominently enriched by GFP-CCA1/LHY, but not in the Col-0 control (Fig. 4c). Perhaps the subtle differences between ChIP-qPCR data and EMSA results are due to the heterodimers formed by CCA1 and LHY *in vivo*, thus co-regulating downstream targets [19,20].

Additionally, TIC was reported to repress other far-red light-signaling components including *FHY1* and *FHL* [12]. Whether CCA1 and LHY are also involved in this process, we first examined the transcript levels of *FHY1* and *FHL* in *cca1-1*, *lhy-20*, and *cca1-1 lhy-20* mutants at pre-dawn and post-dusk. In this, we found that the expression of *FHY1* and *FHL* also increased in the double mutant and predominantly increased at pre-dawn, not post-dusk (Supplementary Fig. 7a), implying CCA1 and LHY worked redundantly with TIC in repressing other far-red light signaling factors. Furthermore, the analysis of *FHY1* and *FHL* promoters were conducted and several

cis-elements were discovered (Supplementary Fig. 7b). EMSA assays demonstrated that GST-CCA1 and GST-LHY, but not GST control, directly bound to the promoters of *FHY1* and *FHL*, containing EE element (EE, AAAATATCT), EE-like element (EEL, AATATCT) and CCA1-binding site (CBS, AAAAATCT) (Supplementary Fig. 7c). ChIP-qPCR assays with GFP-CCA1/LHY transgenic seedlings collected at ZT0 validated these results. The relative enrichments normalized by *APX3* data displayed significant enrichment at the amplicons of EE elements or CBS in the *FHY1* and *FHL* promoters by GFP-CCA1/LHY (Supplementary Fig. 7d).

Given that CCA1 and LHY exhibit transcriptional inhibition and binding to *PHYA* and other far-red light signaling components in the morning, we further investigated the roles of TIC-CCA1/LHY complex using a transient expression assay in *Nicotiana benthamiana* leaves. *PHYApro:LUC* co-expressed with GFP-CCA1/LHY or GFP-TIC and GFP-CCA1/LHY in 5-week *N. benthamiana* leaves, with *pGUS-HA* as a reference plasmid, found that the expression of GFP-CCA1/LHY can repress the promoter activity of *PHYA* relative to GFP control (Fig. 4d-f). Moreover, bioluminescence signals of the *PHYA* promoter were further weakened by co-infiltrating GFP-TIC and GFP-CCA1/LHY (Fig. 4d-f). To validate these findings in Arabidopsis, we performed similar transient expression assays using protoplasts derived from Col-0 and the *tic-2* mutant. Expression of CCA1-HA and LHY-HA in Col-0 protoplasts markedly repressed *PHYA* transcription, whereas these repressions were attenuated in *tic-2* protoplasts (Supplementary Fig. 8). These results suggest that TIC may enhance the transcriptional repression of *PHYA* by CCA1/LHY.

To further explore how TIC and CCA1/LHY functionally contribute to each other's repression of *PHYA* transcription, we employed a transient expression system in *Nicotiana benthamiana* to assess whether co-expression of GFP-TIC could affect the binding of CCA1/LHY to the *PHYA* promoter. The results showed that both experimental groups exhibited clear enrichment at the S4 and S5 regions of the *PHYA* promoter compared to the control, which is consistent with our earlier observations. However, the binding of LHY-HA to the *PHYA* promoter in the presence of GFP-TIC was comparable to that observed when co-expressed with the negative control

GFP-TOC1 (Supplementary Fig. 9a). To ensure the validity of the results, we confirmed comparable protein expression levels across these samples, thereby minimizing potential bias due to differential protein accumulation (Supplementary Fig. 9b). Based on these findings, we propose that TIC is unlikely to enhance CCA1/LHY-mediated repression of *PHYA* solely by increasing their recruitment to the *PHYA* promoter. Conversely, we performed ChIP assay in Arabidopsis using the *TGT*/*lhy-20* seedlings to evaluate whether CCA1/LHY are required for TIC association with the *PHYA* promoter. The results showed that TIC enrichment at the S5 region was not significantly altered in the absence of LHY (Supplementary Fig. 9c), suggesting potential functional redundancy between CCA1 and LHY. Nonetheless, we cannot rule out the possibility that TIC may still associate with the *PHYA* promoter independently of both CCA1 and LHY, as TIC might function as a scaffold protein that recruits many other transcription factors.

TIC is epistatic to CCA1/LHY in mediating FR-repressed hypocotyl elongation

Our findings demonstrated that CCA1 and LHY can interact with TIC to negatively regulate *PHYA* and other far-red light-signaling components by binding to their promoters. Since the *cca1-1 lhy-20* double mutant demonstrated a modestly shortened hypocotyl length under far-red light, which is similar to *tic* mutants, we sought to investigate the genetic relationship between TIC and CCA1/LHY. Therefore, we obtained *cca1-1 lhy20 tic-2* triple mutant after genetic crossing and then examined the hypocotyl growth of *cca1-1 lhy-20*, *tic-2*, and *cca1-1 lhy-20 tic-2* in response to FRc. Consistent with our previous report, *tic-2* mutant seedings displayed significantly shortened hypocotyls under FRc [12], while *cca1-1 lhy-20* showed slightly shorter hypocotyls than Col-0 (Fig. 5a, b). Importantly, the hypocotyl length of *cca1-1 lhy-20 tic-2* in FRc was comparable to that of *tic-2* mutant (Fig. 5a, b), suggesting TIC is epistatic to CCA1/LHY in mediating FR-repressed hypocotyl elongation. Moreover, we examined the hypocotyl phenotypes of these relevant mutants under continuous light, long-day, and short-day conditions at a light intensity of 40 μmol m^{-2} s^{-1}. The *cca1-1 lhy-20 tic-2* triple mutant exhibited a significantly shortened hypocotyl under

all tested light conditions, without showing an additive effect (Supplementary Fig. 10). Notably, under short-day condition, its phenotype closely resembled that of the *cca1-1 lhy-20* double mutant (Supplementary Fig. 10). These results suggest that the Morning Complex may act through a distinct mechanism to coordinately regulate hypocotyl elongation in a photoperiod-independent manner. Finally, we examined the transcript levels of *PHYA*, *FHY1*, and *FHL* in *cca1-1 lhy20 tic-2* at ZT0 and ZT12. As previously detected, *PHYA* and *FHY1* transcripts markedly increased in *cca1-1 lhy-20* and *tic-2* mutants at ZT0, and *FHL* showed a modest rise in *cca1-1 lhy-20*, roughly one-fold higher than in Col-0 at ZT0 (Fig. 5c). Such alterations were restricted to ZT0 and were absent at ZT12 (Fig. 5c). These transcript levels in *cca1-1 lhy-20 tic-2* triple mutants at ZT0 were also significantly increased, which were consistent with those in *tic-2* (Fig. 5c). Notably, *FHY1* expression in the triple mutant was more similar to that in *tic-2* than to the higher levels seen in *cca1-1 lhy-20*, suggesting that TIC may play a more dominant role than CCA1/LHY in regulating genes involved in far-red light signaling. Furthermore, *FHY1* expression at ZT12 was significantly reduced in both *tic-2*, implying that TIC may also facilitate gene activation at certain time points, possibly through the recruitment of other transcription factors or chromatin-modifying components. Collectively, these results suggested that the transcriptional inhibition of these genes by TIC and CCA1/LHY are in the same pathway at pre-dawn. The inference was further confirmed by the expression of other genes co-regulated by the complex in the *cca1-1 lhy-20 tic-2* triple mutants, such as *PRR7*, *CBP1*, *DREB2A*, *DREB2B*, and *FKF1* (Supplementary Fig. 11). In contrast, *PRR5* expression level in *cca1-1 lhy-20 tic-2* triple mutants displayed an additive patter at ZT0 (Supplementary Fig. 11), which supports that TIC and CCA1/LHY play different roles in inhibiting a subset of given gene targets.

Overall, we concluded that TIC-CCA1/LHY functions as a "morning complex", repressing the expression of many genes involved in key biological processes, notably those in morning-related light perception. *PHYA* and its signaling pathway components are the main targets of the morning complex, providing the mechanism of circadian gating of FR regulation at dawn, thereby functioning as negative regulators

of hypocotyl photomorphogenesis under constant far-red light (Fig. 5d). To explore whether the morning complex might function in a broader range of plant species, we conducted further investigations The evolutionary relationships analysis of TIC, CCA1, and LHY revealed that all three genes originated early in land plant evolution, and these genes are highly conserved among angiosperms and exhibit clear divergence between monocot and dicot lineages (Supplemental Fig. 12a and b). Then we found CCA1/LHY showed a conserved dawn peak in Arabidopsis, alfalfa, maize, and rice, whereas TIC displayed relatively constant expression levels throughout the day (Supplemental Fig. 13). These patterns suggest a conserved co-expression and potential functional association between TIC and CCA1/LHY in diverse plants. Although it remains unclear whether TIC possesses conserved functional domains, some of its functions appear conserved in crops. For instance, TIC regulates jasmonic acid (JA)-mediated biotic stress responses in Arabidopsis, and this role is maintained in monocot species. Moreover, the structural domains of CCA1 and LHY are highly conserved across diverse plants and remain functionally involved in circadian regulation and related biological processes [40,41]. Collectively, these findings suggest that TIC and CCA1/LHY likely participate in a conserved circadian clock–mediated light signaling pathway across species.

Discussion

Circadian clocks are evolutionarily conserved molecular mechanisms that synchronize physiology and metabolism with the external environment. The crosstalk between circadian components and light signaling is critical for plants growth. Here, we demonstrated that there is a morning complex that co-regulates various biological processes, and plays a crucial role in regulating light response at dawn. *PHYA* and its signaling components are major repressive targets of the morning complex that mediates its activation of FR-inhibited hypocotyl growth. The morning complex, together with light, PIFs, and other regulatory factors, coordinates a rhythmic transcriptional pattern in which *PHYA* mRNA levels are subsequently downregulated during the day, and gradually rise to a peak just before dawn, thereby enabling PHYA to act as a dawn and photoperiod receptor.

Our study showed that CCA1 and LHY possess transcriptional inhibitory ability on numerous targets (Fig. 2). However, it has been debated whether CCA1/LHY act as the transcriptional repressors or activators. The phenotypes of *CCA1-SRDX* which produce a dominant negative transcriptional repressor and *CCA1-OE* transgenic plants are not exactly the same [43], and in rice, OsCCA1 has been reported to induce ABA signaling components through binding to their promoters [44]. Consistent with this complexity, our results show that loss of *TIC* alone has a stronger impact on *PRR7* than the loss of *CCA1/LHY* or all three factors combined (Supplementary Fig. 11). This observation raises the intriguing possibility that, although CCA1/LHY normally repress *PRR7* at ZT0, they may act as positive regulators of *PRR7* at the same time independent of TIC. Thus, while the mechanism underlying the transcriptional function of CCA1/LHY remains unclear, we propose that their activities may depend on the partners within the transcriptional complex.It was reported that CCA1/LHY are components of a 440 kD complex in plants [19]. Here, we reported that CCA1 and LHY interact with TIC (Fig. 1), which also interact with co-repressor TPL [12,45,46]. As TIC is about 165 kD by itself, and that CCA1 and LHY would be dimers of ~140 kD, and TPL is about 125 kD, thus a TIC/TPL/CCA1/LHY-complex is very close to 440 kD.

Although CCA1 and LHY do not contain the EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif (LxLxL) which can be an interaction motif to interact with TPL [45], we could readily detected their interactions through Co-IP assays (Supplementary Fig. 14). One possibility is that TIC functions as a bridge the two parts to constitute a complete co-inhibitory complex that functions at dawn (Fig. 5c). In that, TIC coordinates transcriptional repression, akin to the role of ELF3 in the evening complex. Moreover, we observed that H3K9 acetylation at the MC-binding regions of the *PHYA* promoter was significantly elevated in *cca1 lhy* and *tic* mutants compared to Col-0 (Supplementary Fig. 15a), suggesting that the recruitment of TPL may render histone acetylation dynamics essential for the transcriptional repression activity of the morning complex. We next examined the effects of TSA treatment on *PHYA* transcript levels at pre-dawn and hypocotyl elongation under far-red light. In Col-0, TSA significantly induced *PHYA* expression and suppressed hypocotyl growth. Although similar trends were observed in *cca1-1 lhy-20* and *tic-2*, both the induction of *PHYA* and the inhibition of hypocotyl elongation were markedly weaker (Supplementary Fig. 15b, d and e), possibly due to inherently higher histone acetylation levels leading to saturated regulation, or it may indicate that the morning complex partially relies on HDAC activity to regulate pre-dawn *PHYA* transcription and far-red light–mediated hypocotyl growth. Regardless, TPL plays an important role in this process, as the elevated pre-dawn *PHYA* expression and shortened hypocotyl phenotype under far-red light in the *tpl-1* mutant were not significantly affected by TSA treatment (Supplementary Fig. 15c, f and g), indicating that TPL, the MC component, is essential for HDAC-mediated transcriptional repression and hypocotyl regulation.

Previous studies have suggested that regulators such as HY5, JAZ1, and CCA1 modulate downstream far-red light signaling indirectly by interfering with FHY3's transcriptional activation of *FHY1/FHL*[29,47,48]. In contrast, our findings reveal that CCA1/LHY can directly bind to the promoters of *PHYA*, *FHY1*, and *FHL* to repress their transcription, indicating a direct regulatory role that was previously unrecognized. Besides, both CCA1/LHY and phyA can interact with TIC, although

the interaction regions are different. And it is unknown whether CCA1/LHY is involved in TIC regulation of phyA abundance and photobodies formation. Hence, the significant reduction of phyA-GFP photobodies in CCA1-OE etiolated seedlings may not only because of decreased *FHY1* level caused by FHY3 [29]. Collectively, it will be a fascinating work to analyze the composition and formation of phyA photobody like phyB, which could help to further understand this interaction network between light and the clock.

Flowering is a vital physiological process in plants. The early-flowering phenotypes of *cca1 lhy* double mutants imply that CCA1 and LHY play negative factors in flowering regulation [49], while TIC positively regulates flowering, due to the late-flowering phenotypes of *tic* mutants [12,14], suggesting that they play opposite roles in regulation of flowering. However, decreased expression of *LHY* in *tic* mutant provides a possibility for TIC to mediate flowering through positive regulation of CCA1/LHY [13], which may not be a direct transcriptional pathway, but by affecting CCA1/LHY protein stability. The self-inhibition of CCA1/LHY makes this hypothesis reasonable. The materials of *GFP-CCA1/LHY* expressed in *tic* mutant are crucial to examine their genetic relationship in flowering regulation.

Given that the morning complex has been shown to participate in regulating circadian rhythms, modulating light signaling, coordinating growth and development, and mediating stress responses (Fig. 2b), it will be critical to investigate how these processes are balanced in diverse environmental contexts. For example, MdTIC is essential for apple trees to tolerate freezing by mediating cold-responsive gene expression and fatty acid composition [50], and the biochemical role of TIC in mediating disease resistance to biotrophs is conserved in grasses through the precise modulation of jasmonate signaling [51]. Considering that the morning complex may be conserved across different plant species (Supplemental Fig. 12 and 13), there is great potential to characterize the TIC-CCA1/LHY regulatory module in various crops. This module could act as a central cellular hub, integrating external signals to fine-tune growth and enhance stress tolerance. Future research on this complex may provide the foundation for innovative crop design strategies aimed at significantly

enhancing environmental adaptation, improving stress resilience, and increasing yield in changing climates

Materials and methods

Plant Materials and Growth Conditions

The Columbia (Col-0) ecotype was used as the wild type (WT) in this study. The *tic-2* mutant was a T-DNA insertion mutant described previously [13], and *tic-3* was a 1bp deletion mutant generated by CRISPR/Cas9 [12]. The *cca1-1*, *lhy-20* single and double mutants [52], were obtained from Xiaodong Xu (Henan university), with *cca1-1* in a Ws background [53], backcrossed six times with *lhy-20* in a Col-0 background [3]. The *cca1-1 lhy-20 tic-2* triple mutant was generated by crossing *tic-2* to *cca1-1 lhy-20* and confirmed genotypically.

The normal growth condition was 12-h light/12-h dark, white light (200 μmol m^{-2} s^{-1}) and 22 ºC. Hypocotyl length assays were conducted according to a previous study [12]. Seeds placed on half strength of Murashige and Skoog (MS) medium (Phytotech M524) containing 1% sucrose after surface sterilizing, and stratified at 4 ºC for 3 days. After germination induction with 7-9 h white light (200 μmol m^{-2} s^{-1}) exposure, seeds were transferred to constant far-red light conditions (FR∼0.1, 0.3, 0.5, and 1 μmol m^{-2} s^{-1}) for 5 days. Hypocotyl phenotypes were captured by a camera (Canon) and measured through NIH ImageJ software (http://rsbweb.nih.gov/ij/)[54].

Constructs

The constructs of *35S:GFP-TIC*, *35S:GFP-TIC-NT*, *35S:GFP-TIC-CT*, *2YN-TIC*, and *PHYApro:LUC* were described previously [12].

To produce *35S:GFP-CCA1* and *35S:GFP-LHY* transgenic plants, the respective PCR fragments were subcloned into the *Kpn* I and *Xho* I sites of the *pENTB2B* vector, then subcloned into the *35S:GFP-MDC45* vector by LR reaction (11791020, Invitrogen & trade). Then transformed into plants through the floral dip method [55]. To generate the *pCsVMV:CCA1-HA* and *pCsVMV:LHY-HA* constructs, the two fragments were amplified by PCR and subcloned into the *Pst* I and *Kpn* I sites of the *pCsVMV:-HA-1300* vector [45]. To generate *CCA1:CCA1-HA* and *LHY:LHY-HA* constructs, the respective full-length coding sequence were amplified and inserted the *Kpn* I and *Bam*H I sites of the no-promoter *pHA-1300* vector. The fragments of *CCA1*

promoter (-1134 to -1 bp, upstream of the start codon) and *LHY* promoter (-1676 to -1 bp, upstream of the start codon) were subcloned into *Eco*R I and *Kpn* I sites or *Sal* I and *Kpn* I sites of the vectors conducted before, using T4 DNA ligase (EL0011, Thermo Fisher), respectively.

To conduct *35S:CCA1-nYFP*, *35S:LHY-nYFP*, *35S:CCA1-cYFP*, *35S:LHY-cYFP*, *35S:TIC-N-cYFP*, and *35S:TIC-N-cYFP* for BiFC assays, the respective PCR fragments were amplified and inserted into the *Pac* I and *Spe* I sites of the *2YC_pBI* and *2YN_pBI* vectors.

Co-immunoprecipitation (Co-IP) assays

For Co-IP assays, Agrobacteria harboring *CCA1:CCA1-HA* and *LHY:LHY-HA* were transiently expressed individually or co-expressed with *35S:GFP-TIC*, *35S: GFP-TIC-NT*, or *35S:GFP-TIC-CT* respectively in the 4-week-old *N. benthamiana* leaves, and the samples were harvested after 3 days, Protein extraction was conducted on the basis of the method described previously [45]. Protein A agarose beads (15918014, Lablead) with GFP Monoclonal Antibody (A-11120, Thermo Fisher Scientific) were used for immunoprecipitation. The protein supernatant was incubated with the beads for 1 h at 4^{o}C on a rotator, then washed at least 4 times with low-speed centrifugation. The washed beads were re-suspended with protein loading buffer and elute with vigorous shaking several times.

For Co-IP assays of TPL-FLAG with CCA1-HA or LHY-HA, plus GFP-TIC, the infiltrated leaves were cross-linked with 1% formaldehyde for 5 min before grinding. HA-Nanobody-Magarose Beads (KTSM1335, AlpaLifeBio) were used for incubating with the protein supernatant for at least 2 h at 4^{o}C on a rotator. And washed beads 5 times. The rest is consistent with the above method.

Immunoblot was detected with GFP (ab6556, Abcam), HA (11867423001, Roche), FLAG (M20008L, Abmart), and CCA1 (PHY7501S, PhytoAB) antibodies.

Split-luciferase (SLC) assays

The full-length coding sequence of TIC, truncated TIC-NT and TIC-CT fragments were amplified and inserted into the *pCAMBIA1300-cLUC* vector with *Kpn* I and *Sal*

I sites, and the sequences of CCA1 and LHY were cloned into *pCAMBIA1300-nLUC* vector with the same sites. Pairwise constructs were co-infiltrated into 4-week-old *N. benthamiana* leaves transiently. After 3 days, these leaves were immersed into the luciferin buffer for 1 min, and the luminescence signals were captured by a CCD camera (LN/1300-EB/1, Princeton Instruments).

Bimolecular fluorescence complementation (BiFC) assays

For the BiFC assays, Agrobacterium containing the indicated plasmids were co-infiltrated into 4-week-old *N. benthamiana* leaves, and *H2B-mCherry* was used as a nuclear marker. After incubation 3 days, the signals were captured by a confocal microscope (Olympus FV1000MPE).

RNA Extraction and quantitative reverse transcription PCR (qRT-PCR) assays

Samples were collected at the indicated time, placed into the liquid nitrogen, and ground into powder before extracting. RNA extraction and qRT-PCR were performed as described previously [56]. Briefly, total RNA was extracted by using TRIzol reagent (Invitrogen). PrimeScript®RT reagent Kit (TaKaRa) was used to reverse-transcribe RNA into cDNA. qRT-PCR was conducted with Real-time PCR SYBR Green Master Mix (Toyobo, Japan). The parameters of PCR cycling were: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s, followed by melting curve analysis. Gene expression levels were normalized by the geometric mean of *ACTIN2* (*AT3G18780*) and *PP2A* (*AT1G69960*) expression. Experiments were repeated with at least two biological and two technical replicates. Primers used in the assays are listed in Supplementary Table 1.

Transcriptional repression activity assays in tobacco

Agrobacteria carrying various fusion expression vectors were co-infiltrated into 5-week-old *N. benthamiana* leaves via syringe infiltration, with *p35S:GUS-HA* as the reference plasmid. Bioluminescence signals were detected after 3 days with a CCD camera (LN/1300-EB/1, Princeton Instruments). Bioluminescence intensities of the LUC signals were measured by MetaMorph Software (Molecular Devices).

Purified GST-tagged CCA1 and LHY proteins

The GST–CCA1 and GST-LHY fusion proteins were expressed in *Escherichia coli* strain BL21 and purified according to the published protocol [56]. Briefly, all of the fused proteins and GST protein alone used in these assays were induced overnight at 16°C with 1 M isopropyl-*β*-d-thiogalactopyranoside (IPTG), and the GST-tag Purification Resin (P2251, Beyotime) was used to incubate these sonicated proteins at 4°C for 3 h. The GST-resin was washed with washing buffer at least 4 times and eluted with a reduced glutathione solution, thus GST-CCA1 or GST-LHY protein solution obtained.

Electrophoresis mobility shift assays (EMSA)

The EMSA assays were conducted with the LightShfit Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to its instructions. 6-μL-purified GST-CCA1 or GST-LHY or GST protein mingled with 0.5 μL of each biotin-labeled probe into a 20 μL reaction and incubated at 4°C for 1 h. A 100x unlabeled probe (0.5 μL) functioned as a competitor was superadded in each reaction. Probes sequence used in EMSA are listed in Supplementary Table 1.

ChIP-qPCR assays

All ChIP experiments were performed following established protocols as previously described [57]. Two-week-old seedlings, grown on MS medium supplemented with 3% sucrose and 0.8% agar at 22 °C under 12-hour light/12-hour dark conditions, were harvested at ZT0 (pre-dawn) for Arabidopsis ChIP assays. GFP-Nanobody Magnetic Beads (GNM-25-1000, Lablead) were used for immunoprecipitation in experiments involving Col-0, GFP-CCA1, and GFP-LHY lines. H3 (ab1791, Abcam) and H3K9ac (ab10812, Abcam) antibodies were used assess histone acetylation levels at the *PHYA* promoter binding regions in Col-0, *cca1 lhy*, and *tic-2* backgrounds. The ChIP assay with *N. benthamiana* leaves was performed based on published study[58], HA antibody (ab9110, Abcam) was used for immunoprecipitation. Input and ChIPed DNA were used for qPCR. The methods for calculating relative enrichment were provided in the corresponding figure legend. All the primers used in the assays are listed in

Supplementary Table 1.

Phylogenetic analysis

Full-length protein sequences of TIC and CCA1/LHY homologs from representative green plant species (*Arabidopsis thaliana*, *Oryza sativa*, etc.) were retrieved from NCBI. These homologues were aligned by MUSCLE. The phylogenetic tree was constructed using MEGA7.0, and evolutionary distances were estimated with a neighbor-joining algorithm. The robustness of each branch was assessed by 1,000 bootstrap replicates. *Physcomitrella patens* homologous protein was used as outgroup.

Quantification and Statistical Analysis

Quantitative data values are defined in the corresponding figure legends, and statistical analysis were performed through SPSS (Statistical Package for the Social Sciences) software (https://www.ibm.com/products/spss-statistics). The paired t-test was used for significance analysis between two sets of data, while one-way ANOVA (Analysis of Variance) followed by Tukey's HSD (honestly significant difference) test, was applied for comparisons among multiple groups, as indicated in figure legends. In all analysis, p value ≤ 0.05 represents statistical significance, and ns means no significance.

Data availability

All other quantitative data generated in this study are published as source data with this article. Source data are provided with this paper.

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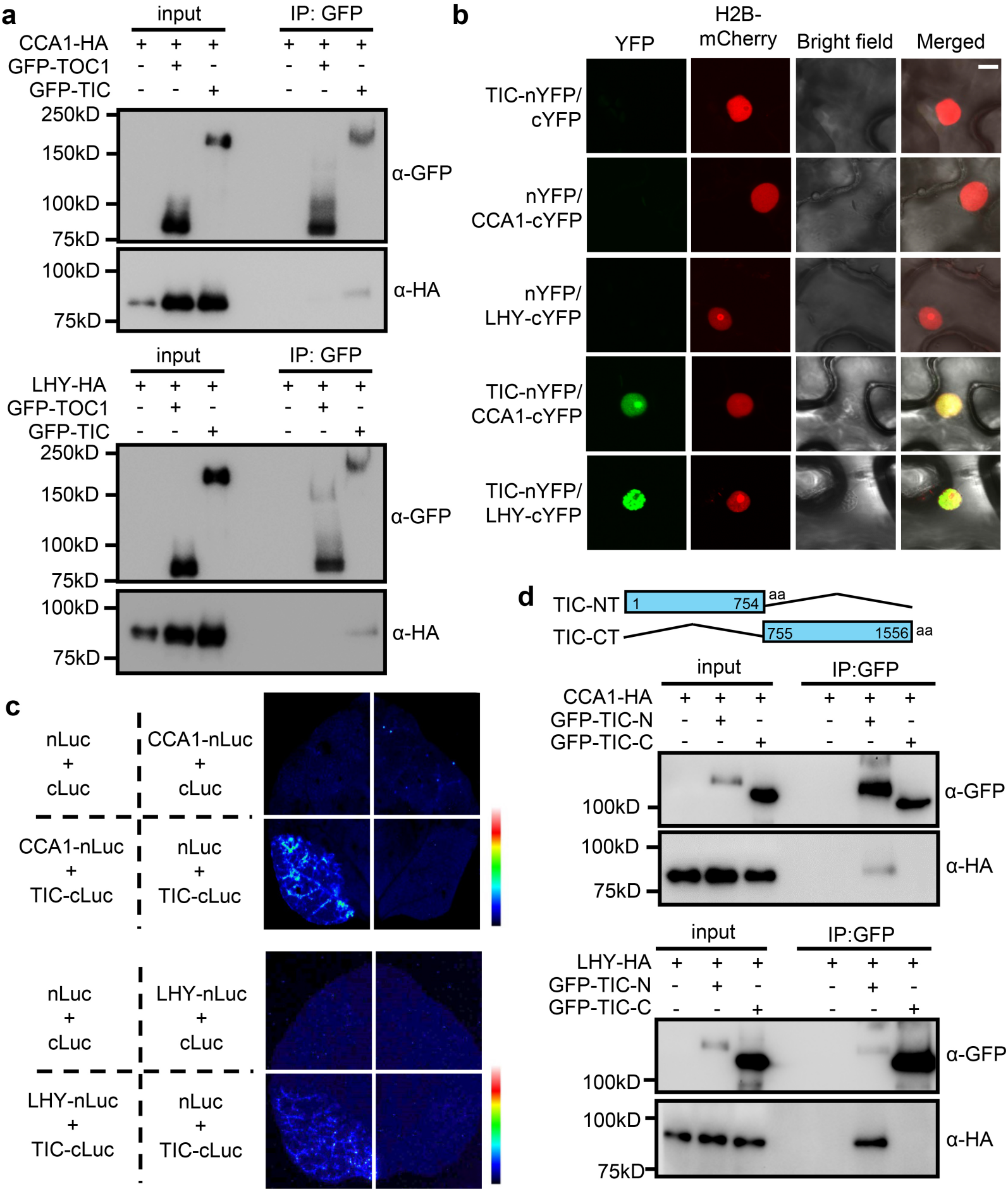
Contributions

C.S., Y.M.Q, Y.Y.J., Y.W., and Y.Q.H. performed the experiments, C.S., S.J.D, and L.W. designed the project, C.S. analyzed the data and wrote the article, S.J.D. and L.W. revised the article. S.J.D. and L.W. agree to serve as the co-author responsible for contact and ensure communication.

Ethics declarations

Competing interests

The authors declare no competing interests.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100kD
75kD
α-HA
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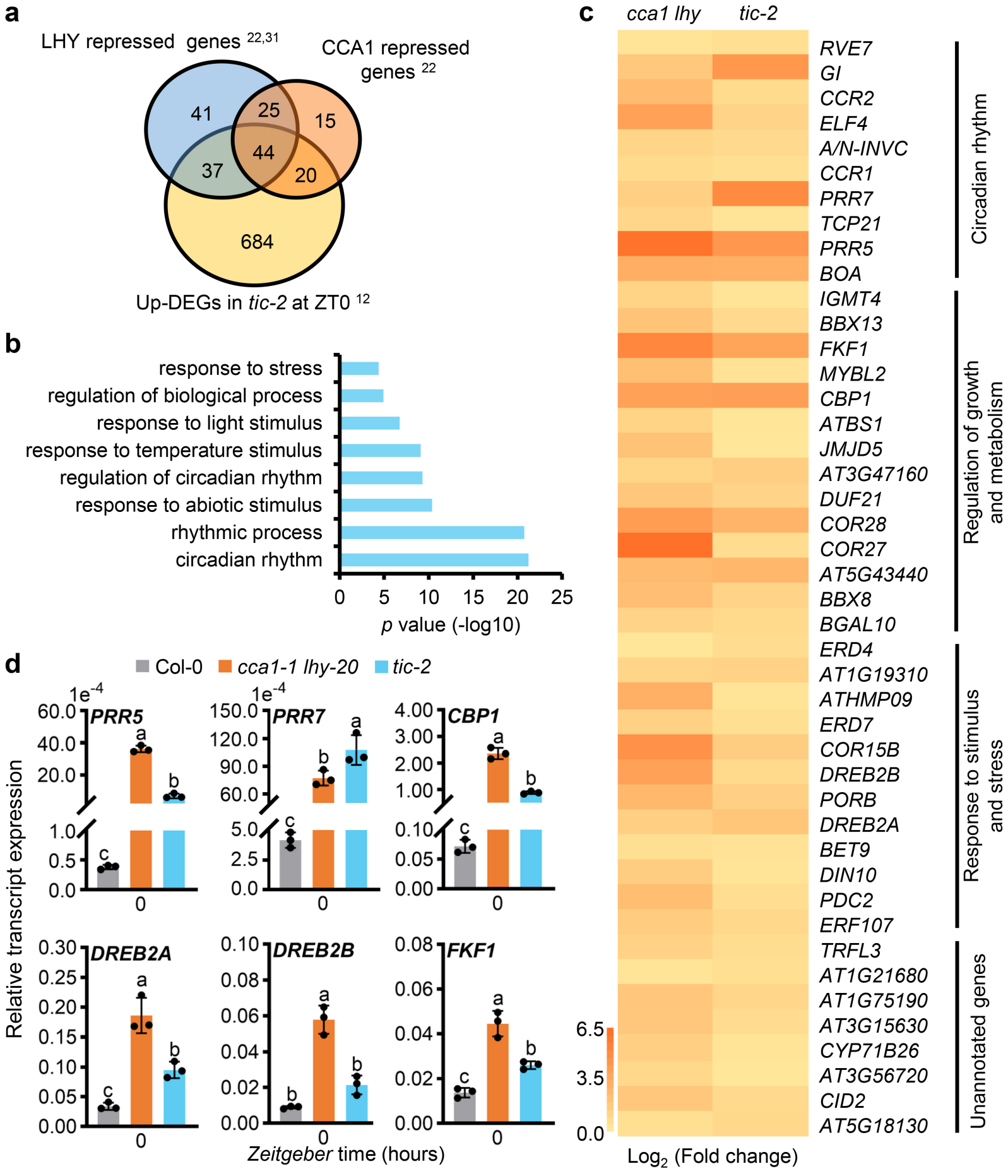
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regulation of biological process
response to light stimulus
response to temperature stimulus
regulation of circadian rhythm
response to abiotic stimulus
rhythmic process
circadian rhythm
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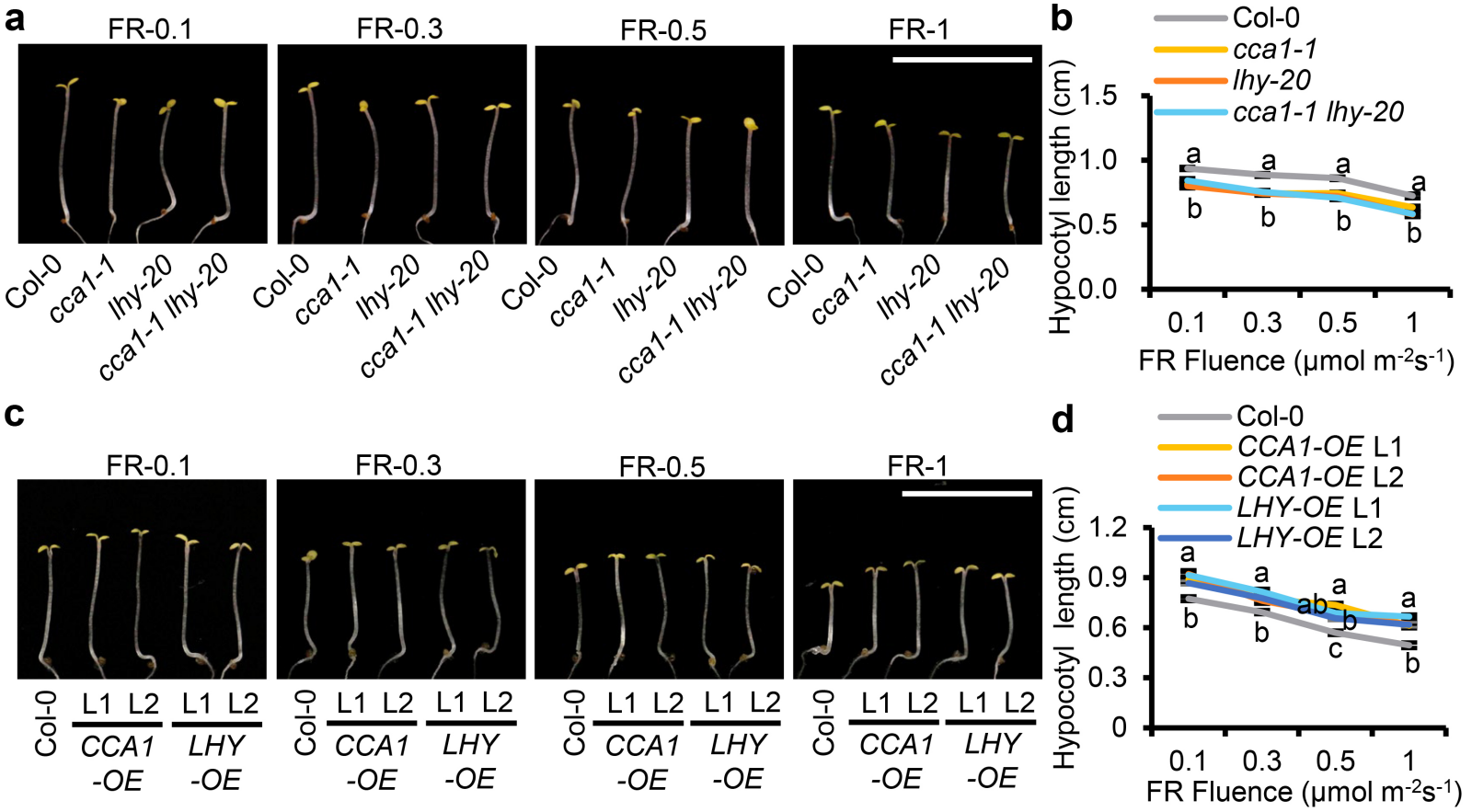
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lhy-20
cca1-1 lhy-20
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1.0
0.5
0.0
0.1
0.3
0.5
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FR-0.3
FR-0.5
FR-1
Col-0
L1 L2
CCA1 -OE
LHY -OE
d
Col-0
CCA1-OE L1
CCA1-OE L2
LHY-OE L1
LHY-OE L2
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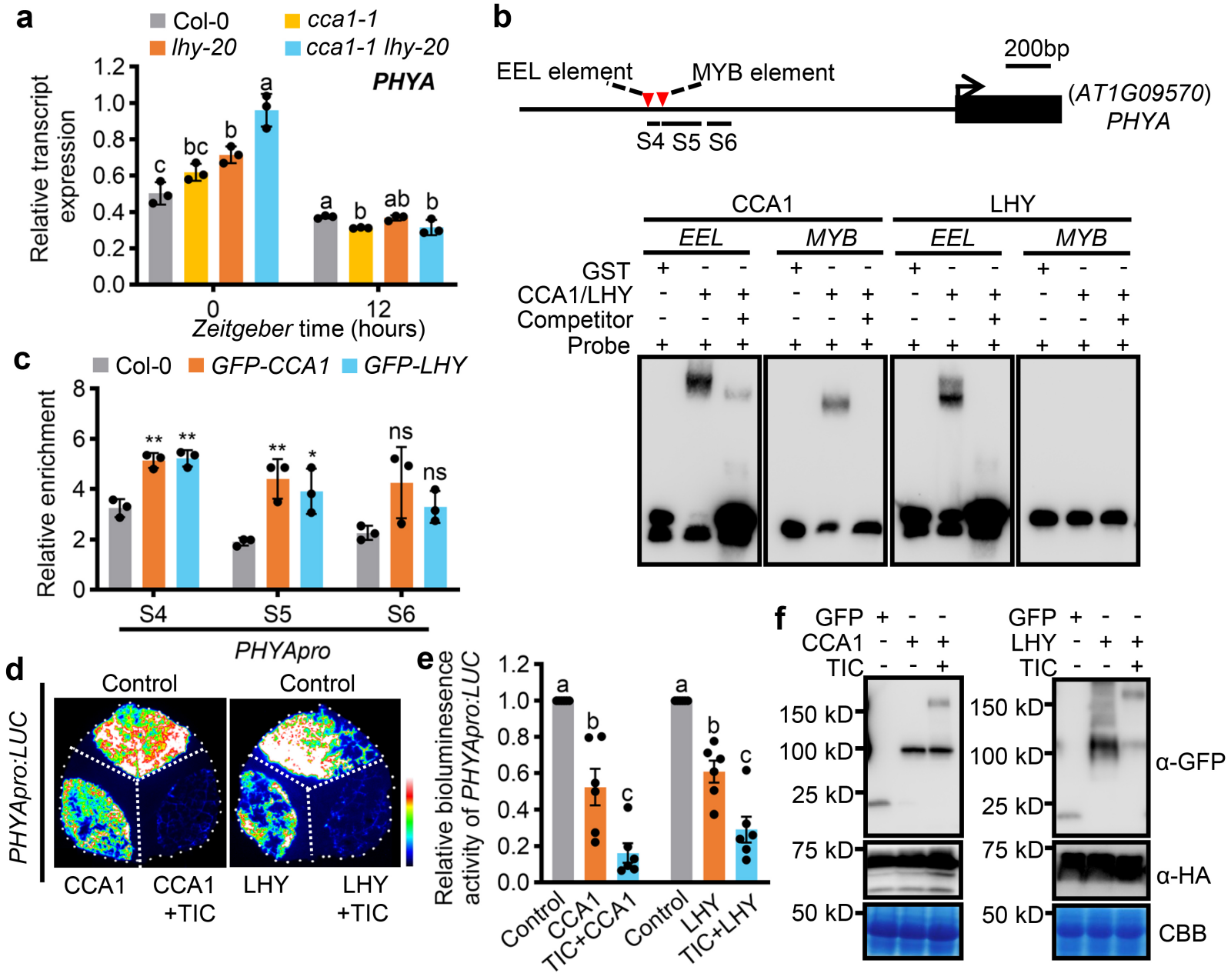
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MYB element
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LHY
EEL
MYB
GST
CCA1/LHY
Competitor
Probe
c
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GFP-LHY
Relative enrichment
S4
S5
S6
PHYApro
d
PHYApro:LUC
Control
Control
CCA1
CCA1 +TIC
LHY
LHY +TIC
e
Relative bioluminescence activity of PHYApro:LUC
Control
CCA1
TIC+CCA1
Control
LHY
TIC+LHY
f
GFP
CCA1
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TIC
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25 kD
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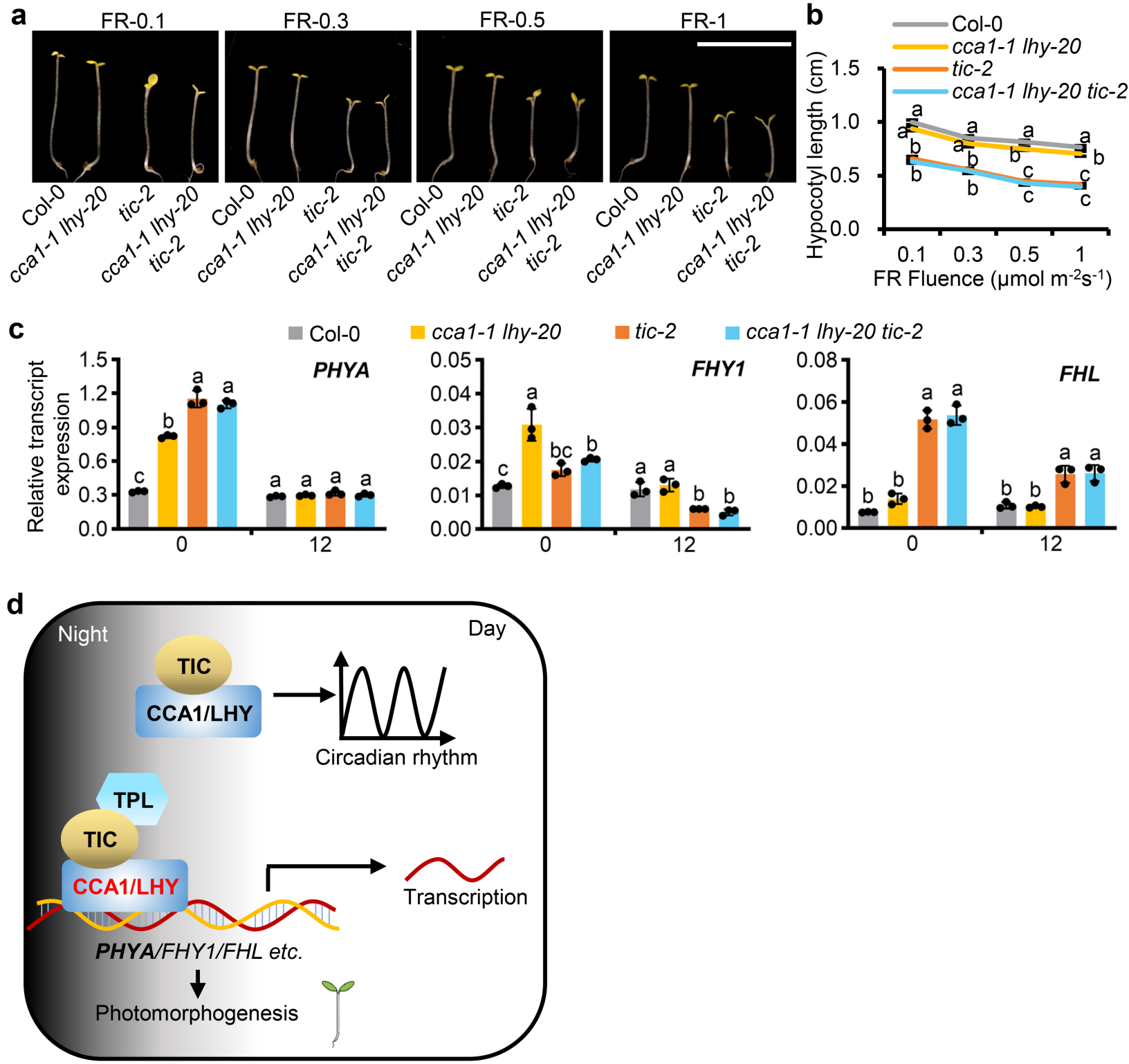
α-HA
CBB


a
FR-0.1
FR-0.3
FR-0.5
FR-1
Col-0
cca1-1 lhy-20
tic-2
cca1-1 lhy-20 tic-2
b
Hypocotyl length (cm)
FR Fluence (μmol m-2s-1)
c
Relative transcript expression
PHYA
FHY1
FHL
d
Night
Day
TIC
CCA1/LHY
Circadian rhythm
TPL
Transcription
PHYA/FHY1/FHL etc.
Photomorphogenesis