nature portfolio

Peer Review File

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

Corresponding Author: Professor Lei Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Su et al. demonstrates for the first time the physical and functional interaction between the plant circadian clock regulator protein, TIC, with the key central circadian clock proteins, CCA1 and LHY within the Arabidopsis clock. The paper describes a very thorough analysis of the physical interaction of the proteins and demonstrates their time of day-specific regulation of light responses during seedling etiolation. Furthermore, another thorough analysis of both mutants and overexpressors demonstrates epistasis between these components in this process. Interaction between these components in the negative regulation of expression of genes controlled by the phyA photoreceptor is also demonstrated. CCA1 and LHY are demonstrated to bind to specific promoter regions of the PHYA gene itself, and of the FHY1 and FHL genes involved in phyA photoreceptor signalling, consistent of their downregulation of expression of these targets. As well as showing strong commonality between TIC- and CCA1/LHY-downregulated gene sets, the authors also demonstrate that TIC protein can enhance CCA1 and LHY-mediated downregulation of PHYA gene expression. Finally, interaction between TIC1 and CCA1/LHY in their time-of day-specific regulation of light responsive gene expression is analysed.

The findings of the manuscript are entirely novel and reveal a significant advance in terms of our understanding of the mechanism by which the plant circadian clock impacts upon plant light responses. The experiments are rigorous, well designed well-presented and, in the main, interpretation of the findings is sound. The paper is also well-constructed and well-written, and the overall conclusions are well-supported.

Points which require addressing:
There are a few discrepancies in the interpretation of the final set of experiments looking at interaction between TIC1 and CCA1/LHY in their time-of day-specific regulation of light responsive gene expression. And a point from the discussion also raises a question. While these do not detract from the overall conclusion, they do need addressing within the text.

Line 243: “the expressions of PHYA, FHY1, and FHL increased in cca1-1 lhy-20 and tic-2 mutants at ZT0, but not at ZT12”. Loss of CCA1 and LHY does not cause any increase in FHL level in this dataset. And, importantly, this contradicts Fig S7a referred to in an earlier section. How do the authors reconcile this?

Lie 244: “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”. Loss of TIC does not seem to further increase FHY1 relative to levels in the cca1 lhy mutant as might be expected. In fact, based on both ZT0 and ZT12 results, it seems TIC has a positive effect on FHY1 levels which also acts contrary to the loss of CCA/LHY at ZT0. Please also add some discussion of these observations.

Line 248: “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”. The pattern in PRR7 actually suggests that loss of TIC is somehow ameliorated by loss of CCA1 and LHY. Please also add some discussion of these observations.

Line 269: “similar to CAB, making it highly expressed at dawn”. The similarity to CAB is only partial. CAB genes are positively regulated by CCA1. Furthermore, CAB is highly expressed mid-morning unlike PHYA which is highly expressed late at night and CAB light activation is maximal at times of high CCA1 protein as shown in the reference cited here. In addition, would repression of PHYA expression by a morning complex not be expected to give a PHYA expression peak at dusk as is seen for many other genes negatively regulated by CCA1/LHY? How is this reconciled with a PHYA peak prior to

dawn?

Minor points:
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Line 119: “interaction intensity with TIC-N seemed to be stronger”. This is true for BiFC but not clear for split LUC.
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Line 250 There should be a comma before “while” not a full stop.
Line 298: “which differs from before”. Please clarify.

Reviewer #2

(Remarks to the Author)
This manuscript presents the identification and functional characterization of a "Morning Complex" (MC) consisting of TIME FOR COFFEE (TIC), CCA1, and LHY within the Arabidopsis circadian system. The authors demonstrated that these components physically interact in the nucleus and act specifically at dawn. In particular, the MC directly binds to the promoters of key far-red light signaling genes such as PHYA, FHY1, and FHL, inhibiting their expression around dawn. This feedback mechanism links clock function to light signaling and contributes to the regulation of hypocotyl elongation under far-red light. Genetic analysis revealed that TIC is epistatic to CCA1/LHY. Overall, while this study provides significant mechanistic insights into clock-light crosstalk in plants, several aspects of the study appear preliminary and would benefit from additional experiments to strengthen the main claims.

Major comments
1. While the physical interactions between TIC and CCA1/LHY were convincingly demonstrated, it remains unclear whether the formation of the Morning Complex is context-dependent. The authors should clarify if TIC is broadly required for CCA1-LHY-mediated functions, beyond far-red signaling. For instance, does TIC influence photoperiod-independent hypocotyl elongation regulated by CCA1 and LHY?
2. The biochemical relevance of the MC formation remains speculative. It would be needed to explore whether TIC enhances the DNA-binding affinity or binding strength of CCA1 and LHY at target promoters (e.g., PHYA), and vice versa.
3. If promoter binding is not substantially altered by complex formation, the MC may function by modulating transcriptional repression capacity. Given TIC’s known interaction with the co-repressor TPL, it is plausible that the MC acts not only through DNA binding-dependent gene repression but also via chromatin-level repression. It would be informative to test whether CCA1/LHY function in far-red signaling depends on TPL and/or HDAC activity—using pharmacological inhibitors or genetic mutants.
4. In Figure 4d–e, transient expression assays using Arabidopsis mesophyll protoplasts would provide more physiologically relevant insights. The authors could test whether transcription repression of PHYA by CCA1 and LHY is impaired in tic mutant protoplasts. It can also be done to overexpress TIC in cca1 lhy mutant protoplasts.
5. To demonstrate the functional interdependence among MC components, genetic crosses such as TIC-OX in cca1 lhy and CCA1-OX in tic backgrounds would be useful. These could reveal whether one component is required for the other to regulate circadian oscillation and far-red signaling.
6. While Arabidopsis is an excellent model, a discussion on whether homologs of TIC, CCA1, and LHY co-express or co-function together in other plant species would enhance the broader relevance of the finding. Sequence conservation and promoter motif analysis across species would be helpful.

Minor comments
1. Figure 1A, 1D: Please include a negative control using proteins known not to interact with CCA1/LHY in the Co-IP assays to confirm interaction specificity.
2. Figure 2C: Consider adding functional category annotations to the heatmap for easier interpretation.
3. Figure 3B, 3D: Clarify the statistical results corresponding to results.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for their careful consideration of my comments. While most of my comments have been addressed to my satisfaction, I feel that some further work is required for the following comments.

1. Regarding the response to my comment on the former line 243, I don’t agree that the authors’ response, alone, resolves this issue without a change in the manuscript.
My comment related to the fact that the compact letter display in Fig 5c does not show a significant difference between Col-0 and the cca1 lhy double mutant at ZT0 for FHL expression as was claimed in the text. (Both receive cld letter b based on a Tukey HSD post-hoc set of pairwise tests according to the legend).
The authors’ suggested that this lack of significance was due to “between group differences”. While this may be the case, the graph presented still shows no significant difference so the description of the results must reflect this.
If the Tukey’s HSD test is not felt to be suitable here, then an alternative statistical text could be applied to the data. The

authors mention in their rebuttal that a single pairwise t-test between these two values in isolation shows significance. However, it is not stated whether this involved any correction for multiple testing (e.g. Bonferroni), which would be required as this is just one of many pairwise t-tests that could be applied to that data. If this still shows a significant difference after Bonferroni correction, then it would be valid for the authors to make the claim, but the test would need to be mentioned in the manuscript (and, ideally, the same the test should also be applied to the rest of Fig5c too).

2. Regarding the response to my comment on the former line 248, I also don't agree that the authors' response fully resolves this issue.
I had mentioned that it looks like the effect of loss of TIC on PRR7 expression at ZT0 is somehow ameliorated by loss of CCA1 and LHY and suggested that this could be discussed. However, I feel that the argument made by the authors in their response - that the regulation of PRR7 by the Morning Complex may be more dependent on CCA1/LHY function - doesn't fully fit the data.
Loss of TIC alone has a much greater impact on PRR7 than either loss of CCA1/LHY alone or loss of all three factors. A possible explanation is that, although CCA1/LHY normally inhibit PRR7 at ZT0, they can also act to positively regulate PRR7 at this time in the absence of TIC. (i.e. TIC changes the sign +/- of the CCA1/LHY effect on PRR7 expression).

3. Regarding the response to my comment on the former line 269, the authors state that they have removed the phrase "similar to CAB" but this still appears in the amended manuscript.
The authors response here that "This [PHYA] expression pattern reflects the outcome of integrated regulatory mechanisms rather than a simple correlation with CCA1/LHY expression dynamics alone" provides a very good alternative explanation to that which appears in the text. It would also be good to include that in the discussion at that point rather than the current text which still just relates PHYA expression level to the action of the morning complex.

All of my other comments have been addressed to my satisfaction.

Reviewer #2

(Remarks to the Author)
Most of my concerns have been addressed during the revision.

Reviewer #1 (Remarks to the Author)

The manuscript by Su et al. demonstrates for the first time the physical and functional interaction between the plant circadian clock regulator protein, TIC, with the key central circadian clock proteins, CCA1 and LHY within the Arabidopsis clock. The paper describes a very thorough analysis of the physical interaction of the proteins and demonstrates their time of day-specific regulation of light responses during seedling etiolation. Furthermore, another thorough analysis of both mutants and overexpressors demonstrates epistasis between these components in this process. Interaction between these components in the negative regulation of expression of genes controlled by the phyA photoreceptor is also demonstrated. CCA1 and LHY are demonstrated to bind to specific promoter regions of the PHYA gene itself, and of the FHY1 and FHL genes involved in phyA photoreceptor signalling, consistent of their downregulation of expression of these targets. As well as showing strong commonality between TIC- and CCA1/LHY-downregulated gene sets, the authors also demonstrate that TIC protein can enhance CCA1 and LHY-mediated downregulation of PHYA gene expression. Finally, interaction between TIC1 and CCA1/LHY in their time-of day-specific regulation of light responsive gene expression is analysed.

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There are a few discrepancies in the interpretation of the final set of experiments looking at interaction between TIC1 and CCA1/LHY in their time-of day-specific regulation of light responsive gene expression. And a point from the discussion also raises a question. While these do not detract from the overall conclusion, they do need addressing within the text.
Response: We are grateful for the reviewer's positive comments. According to your suggestions, we have conducted additional experiments and carefully rephrased the manuscript. Moreover, we have provided the point-by-point responses to your concerns as below.

Line 243: "the expressions of PHYA, FHY1, and FHL increased in *cca1-1 lhy-20* and *tic-2* mutants at ZT0, but not at ZT12". Loss of CCA1 and LHY does not cause any increase in FHL level in this dataset. And, importantly, this contradicts Fig S7a referred to in an earlier section. How do the authors reconcile this?
Response: Thanks for your question. In fact, in both Figure 5c and Figure S7a, the expression level of *FHL* at ZT0 in the *cca1 lhy* double mutant is approximately twice that in Col-0. A *t*-test of *FHL* expression levels between Col-0 and *cca1 lhy* in Figure 5c yielded a *p*-value of 0.0128, indicating a significant difference. Therefore, the loss of *CCA1/LHY* in Figure 5c still results in upregulation of *FHL* expression. The statistical analysis in Figure 5c was affected by excessive between-group differences, which potentially masked the significance of smaller differences between specific pairs of groups.

Lie 244: “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*”. Loss of TIC does not seem to further increase *FHY1* relative to levels in the *cca1 lhy* mutant as might be expected. In fact, based on both ZT0 and ZT12 results, it seems TIC has a positive effect on *FHY1* levels which also acts contrary to the loss of CCA/LHY at ZT0. Please also add some discussion of these observations.

Response: Thanks for your suggestion. We have accordingly added the following discussion to the manuscript. “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 *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, or through other unkown indirect effect. Collectively, these results suggested that the transcriptional inhibition of these genes by TIC and CCA1/LHY are in the same pathway at pre-dawn.”

Line 248: “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*”. The pattern in *PRR7* actually suggests that loss of TIC is somehow ameliorated by loss of *CCA1* and *LHY*. Please also add some discussion of these observations.

Response: Thanks for your suggestion. We have accordingly added the following discussion to the manuscript. “Interestingly, *PRR7* expression in the triple mutant more closely resembled that in the *cca1-1 lhy-20* double mutant, indicating that the regulation of *PRR7* by the Morning Complex may be more dependent on CCA1/LHY function.”

Line 269: “similar to *CAB*, making it highly expressed at dawn”. The similarity to *CAB* is only partial. *CAB* genes are positively regulated by CCA1. Furthermore, *CAB* is highly expressed mid-morning unlike *PHYA* which is highly expressed late at night and *CAB* light activation is maximal at times of high CCA1 protein as shown in the reference cited here. In addition, would repression of *PHYA* expression by a morning complex not be expected to give a *PHYA* expression peak at dusk as is seen for many other genes negatively regulated by CCA1/LHY? How is this reconciled with a *PHYA* peak prior to dawn?

Response: Thanks for your detailed suggestions. We have removed the inaccurate expression "similar to CAB."

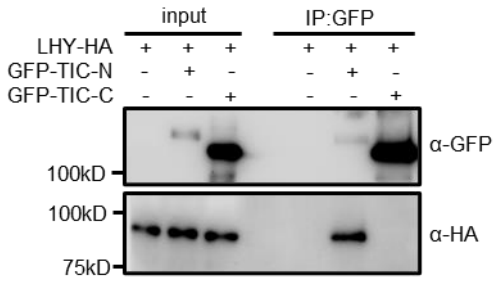
The decline in *PHYA* mRNA levels is driven not only by CCA1/LHY-mediated repression but also by light irradiation, with both phyA and phyB contributing to this light-induced down-regulation[1]. Consequently, *PHYA* transcript levels gradually decrease following light exposure and reach a nadir around dusk. This expression pattern reflects the outcome of integrated regulatory mechanisms rather than a simple correlation with *CCA1/LHY* expression dynamics alone. Moreover, transcriptional activators of *PHYA*, such as PIFs, accumulate during the latter part of the night, leading to a gradual increase in *PHYA* mRNA levels that peak just before dawn[2].

Minor points:

Figure 1d. Please explain the faint band detected by anti-GFP in LHY-HA input only.

Response: Sorry for the confusion. The faint band detected by anti-GFP in LHY-HA input is likely

attributable to technical artifacts, such as spillover from the co-transformation lane or contamination during membrane transfer, so we replaced it with new biological replicate, as shown in the figure below.



Line 119: “interaction intensity with TIC-N seemed to be stronger”. This is true for BiFC but not clear for split LUC.

Response: Thanks for your careful suggestion. We have revised the sentence accordingly. “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”.

Line 129: We compared our up-DEGs of tic-2 at pre-dawn”. Please cite data source here - I believe this is ref 12

Response: Thanks for your reminder. We have added the citation at this point, which indeed corresponds to reference 12.

Line 250 There should be a comma before “while” not a full stop.

Response: Thanks for noticing this detail. We have fixed it.

Line 298: “which differs from before”. Please clarify.

Response: We have revised this as follows: “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*. 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.”. Thanks!

Reviewer #2 (Remarks to the Author):

This manuscript presents the identification and functional characterization of a "Morning Complex" (MC) consisting of TIME FOR COFFEE (TIC), CCA1, and LHY within the Arabidopsis circadian system. The authors demonstrated that these components physically interact in the nucleus and act specifically at dawn. In particular, the MC directly binds to the promoters of key far-red light signaling genes such as PHYA, FHY1, and FHL, inhibiting their expression around dawn. This feedback mechanism links clock function to light signaling and contributes to the regulation of hypocotyl elongation under far-red light. Genetic analysis revealed that TIC is epistatic to CCA1/LHY. Overall, while this study provides significant mechanistic insights into clock-light crosstalk in plants, several aspects of the study appear preliminary and would benefit from additional

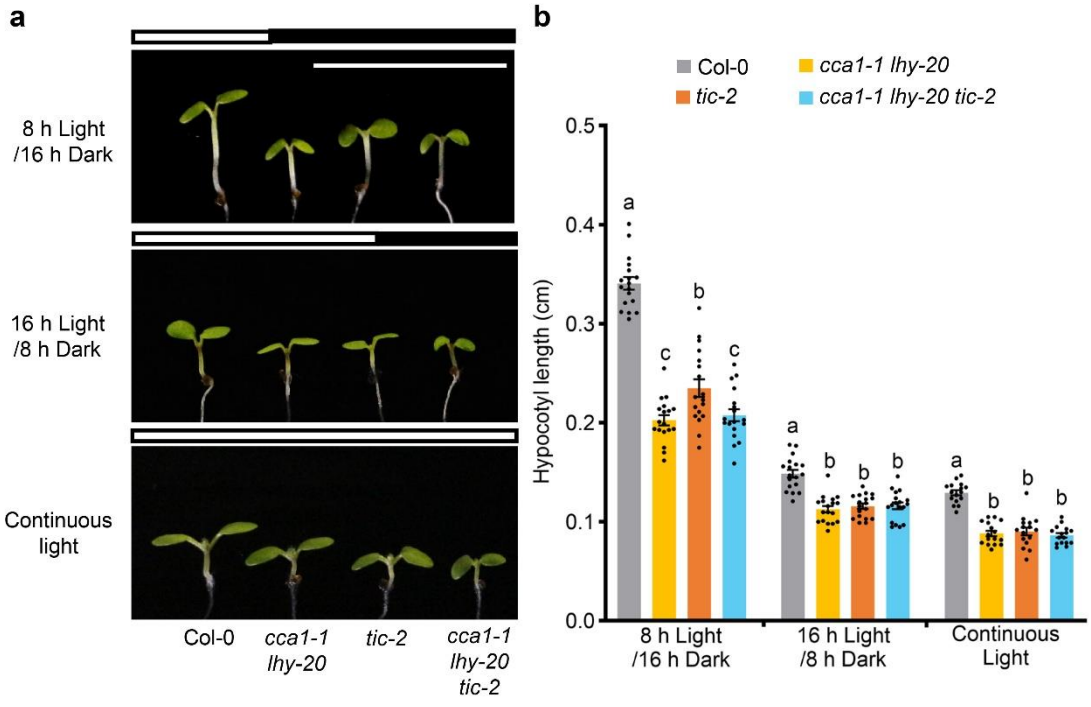
experiments to strengthen the main claims.

Response: We are grateful for the reviewer's positive comments. As suggested, we have performed the additional experiments and carefully rephrased the manuscript. Meanwhile, we have addressed your concerns point-by-point as below.

Major comments

1. While the physical interactions between TIC and CCA1/LHY were convincingly demonstrated, it remains unclear whether the formation of the Morning Complex is context-dependent. The authors should clarify if TIC is broadly required for CCA1-LHY-mediated functions, beyond far-red signaling. For instance, does TIC influence photoperiod-independent hypocotyl elongation regulated by CCA1 and LHY?

Response: Thanks for your helpful suggestion. Although our study mainly focuses on the role of the Morning Complex in regulating the far-red light signaling pathway, it is indeed important to consider its potential involvement in broader biological processes. Previous studies have shown that TIC and CCA1/LHY act within the same genetic pathway to regulate circadian rhythms, with TIC displaying genetic epistasis[3]. As suggested, to investigate whether TIC influences photoperiod-independent hypocotyl elongation mediated by CCA1/LHY, we examined the hypocotyl phenotypes of the relevant mutants under continuous light, long-day, and short-day conditions using 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 and below). Notably, under both long-day and short-day conditions, the hypocotyl growth phenotypes of *cca1-1 lhy-20 tic-2* triple mutant closely resembled those of the *cca1-1 lhy-20* double mutant (Supplementary Fig. 10). These results clearly suggest that TIC influences photoperiod-independent hypocotyl growth by CCA1 and LHY.



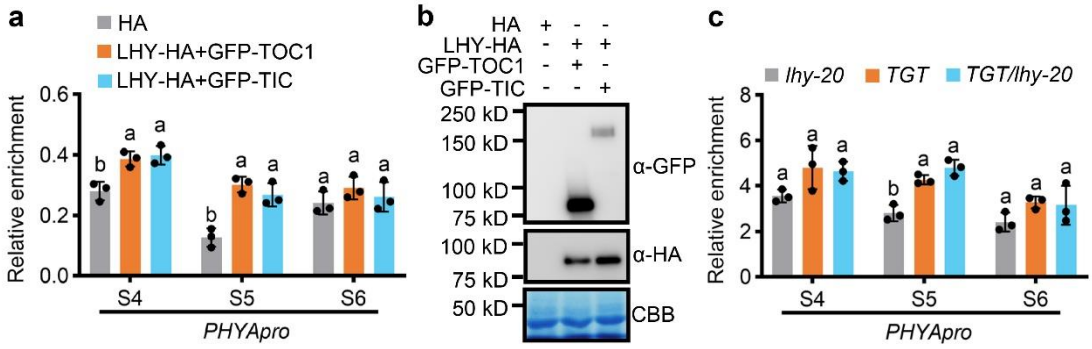
Supplemental Fig 10: Hypocotyl growth of *cca1-1 lhy-20 tic-2* under photoperiodic and continuous white light conditions.

(a) Hypocotyl phenotypes of Col-0, *cca1-1 lhy-20*, *tic-2*, and *cca1-1 lhy-20 tic-2* under SD (8 h L/16 h D), long-day (16 h L/8 h D) and continuous white light conditions (WL ~40 μmol m^{-2} s^{-1}) for 5 days, bar = 10 mm.

(b) Quantitative analysis of hypocotyl length of Col-0, *cca1-1 lhy-20*, *tic-2*, and *cca1-1 lhy-20 tic-2* seedlings tested in (a). Data represent mean ± s.e.m. (n ≥ 15), and lowercase letters indicate significant differences by one-way ANOVA followed determined by Tukey's HSD test ($p \leq 0.05$).

2. The biochemical relevance of the MC formation remains speculative. It would be needed to explore whether TIC enhances the DNA-binding affinity or binding strength of CCA1 and LHY at target promoters (e.g., PHYA), and vice versa.

Response: Thanks for your helpful suggestion. In our proposed model, TIC majorly function as a bridge to recruit TPL and CCA/LHY. To address the effect of TIC on the DNA binding affinity of CCA1/LHY, we employed a transient expression system in *Nicotiana benthamiana* to assess whether co-expression of GFP-TIC could affect the binding of CCA1/LHY on *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 in Arabidopsis. But the binding of LHY-HA to the *PHYA* promoter in the presence of GFP-TIC was just comparable to that observed when co-expressed with the negative control GFP-TOC1 (Supplemental Fig. 9a). To further ensure the validity of the results, we also confirmed the comparable protein levels across these samples (Supplemental Fig. 9b). Based on this evidence, we propose that TIC is unlikely to enhance the binding strength of CCA1/LHY on the *PHYA* promoter. To investigate whether CCA1/LHY are required for TIC binding to the *PHYA* promoter, we performed ChIP assays in *Arabidopsis* using the available *TICpro:GFP-TIC lhy-20* line. The results indicated that TIC enrichment at the S5 region of the *PHYA* promoter was not significantly altered in the absence of LHY (Supplemental Fig. 9c). Nevertheless, we cannot exclude the possibility that TIC may still associate with the *PHYA* promoter even in the absence of both CCA1 and LHY, as TIC might function as a scaffold protein that recruits many other transcription factors.



Supplemental Fig 9: TIC may not enhance CCA1/LHY repression of *PHYA* by increasing their promoter binding.

(a) ChIP assay of LHY-HA co-expressing with GFP-TIC or negative control GFP-TOC1 in *N. benthamiana* leaves. Enrichments were normalized by the mean of *NbACTIN*. Data represent mean ± s.d., and lowercase letters indicate significant differences by one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$).

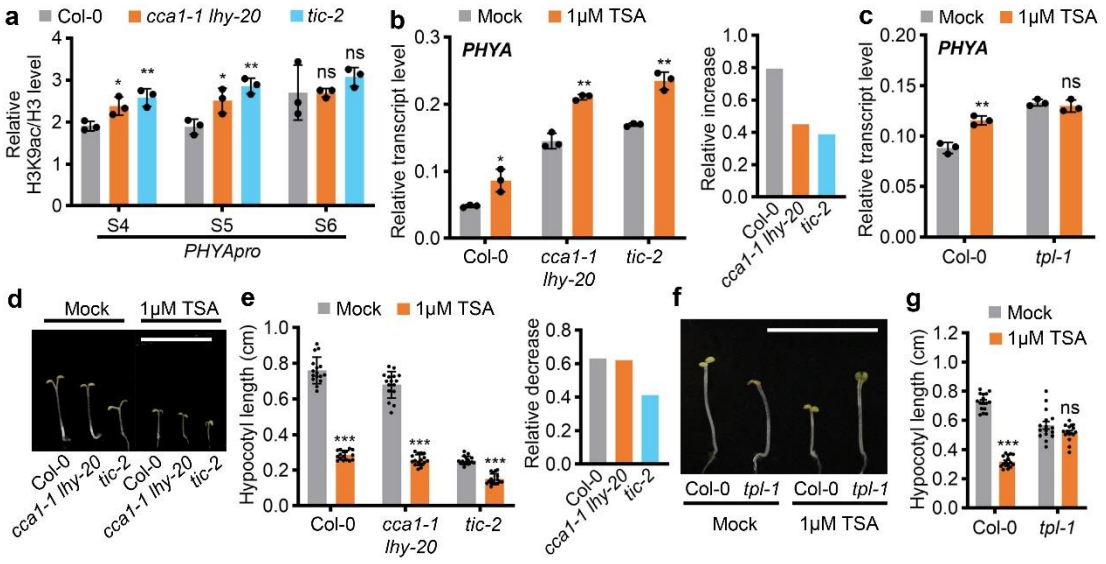
(b) Immunoblot detecting the respective protein levels in (a)

(c) ChIP assay of *lhy-20*, *TICpro:GFP-TIC* (*TGT*), and *TGT/lhy-20* with tissues harvested at pre-

dawn. Enrichments were normalized by the mean of *APX3*. Asterisks indicate significant differences by one-way ANOVA followed by Tukey's HSD test ($p \leq 0.05$).

3. If promoter binding is not substantially altered by complex formation, the MC may function by modulating transcriptional repression capacity. Given TIC's known interaction with the co-repressor TPL, it is plausible that the MC acts not only through DNA binding-dependent gene repression but also via chromatin-level repression. It would be informative to test whether CCA1/LHY function in far-red signaling depends on TPL and/or HDAC activity—using pharmacological inhibitors or genetic mutants.

Response: Thanks for your suggestion. As the involvement of TPL indicates that histone acetylation dynamics are critical for the transcriptional repression function of the Morning Complex (MC), we further examined H3K9 acetylation levels at the *PHYA* promoter regions S4/5/6 at ZT0 in WT, *cca1lhy*, and *tic* mutants. We found that H3K9 acetylation at the MC-binding S4/5 regions was significantly increased in *cca1lhy* and *tic* compared to Col-0 (Supplemental Fig. 15a), suggesting that the recruitment of TPL may render histone acetylation dynamics. Followed your suggestion, we next examined the effects of TSA treatment on *PHYA* transcript levels at pre-dawn and hypocotyl elongation under far-red light. We found 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 (Supplemental Fig. 15b, d and e), indicating that the morning complex at least partially relies on HDAC activity to regulate pre-dawn *PHYA* transcription and far-red light–mediated hypocotyl growth. Notably, TSA treatment had little effect on the elevated *PHYA* expression and shortened hypocotyl phenotype of the *tpl-1* mutant under far-red light (Supplementary Fig. 15c, f and g). Together, these findings support a model in which TPL and histone deacetylase activity are critical for Morning Complex–mediated repression of *PHYA* expression and far-red light signaling.



Supplemental Fig 15: TPL and HDAC activities are critical for MC-mediated repression of far-red light signaling.

(a) Relative H3K9ac/H3 levels in Col-0, *cca1-1 lhy-2* and *tic-2*. Two weeks seedlings entrained in LD cycles were harvested at pre-dawn for ChIP assay with H3K9ac or H3 antibody. Data represent mean ±s.d. Asterisks indicate significant differences according to Student's *t*-test (ns $p > 0.05$, * p

≤ 0.05, ** $p \leq 0.01$).

(b) Transcript levels of *PHYA* at pre-dawn in *cca1-1 lhy-20* and *tic-2* mutants treated with or without 1μM TSA (left panel), normalized to the geometric mean of *ACT2* and *PP2A*. Data represent mean ±s.d. Asterisks indicate significant differences according to Student's *t*-test (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$). Relative increase (right panel) was calculated as (TSA mean – Mock mean)/ Mock mean.

(c) Transcript levels of *PHYA* at pre-dawn in *tpl-1* mutant treated with or without 1μM TSA, normalized to the geometric mean of *ACT2* and *PP2A*. Data represent mean ±s.d. Asterisks indicate significant differences according to Student's *t*-test (ns $p > 0.05$, ** $p \leq 0.01$).

(d) Hypocotyl phenotypes of Col-0, *cca1-1 lhy-20*, and *tic-2* grown under constant far-red light (1 μmol m^{-2} s^{-1}) with or without 1μM TSA for 5 days, bar = 10 mm.

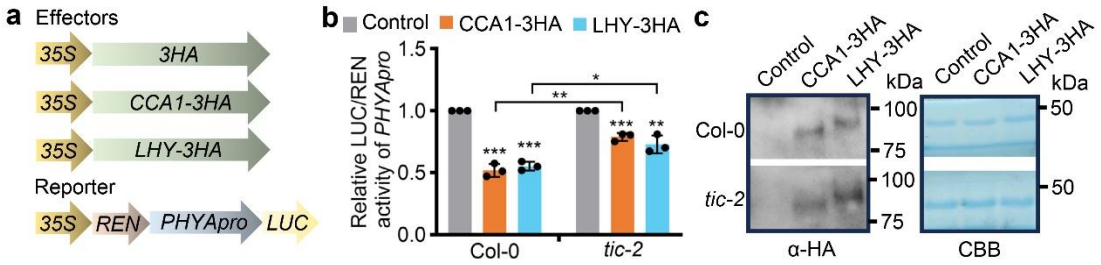
(e) Quantitative analysis of hypocotyl length of Col-0, *cca1-1 lhy-20*, and *tic-2* seedlings tested in (d). Data represent mean ± s.e.m. (n ≥ 16), and lowercase letters indicate significant differences according to Student's *t*-test (*** $p \leq 0.001$). Relative decrease (right panel) was calculated as |TSA mean – Mock mean|/ Mock mean.

(f) Hypocotyl phenotypes of Col-0, and *tpl-1* grown under constant far-red light (1 μmol m^{-2} s^{-1}) with or without 1μM TSA for 5 days, bar = 10 mm.

(g) Quantitative analysis of hypocotyl length of Col-0, and *tpl-1* seedlings tested in (f). Data represent mean ± s.e.m. (n ≥ 16), and lowercase letters indicate significant differences according to Student's t-test (ns $p > 0.05$, ** $p \leq 0.01$).

4. In Figure 4d–e, transient expression assays using Arabidopsis mesophyll protoplasts would provide more physiologically relevant insights. The authors could test whether transcription repression of *PHYA* by CCA1 and LHY is impaired in *tic* mutant protoplasts. It can also be done to overexpress TIC in *cca1 lhy* mutant protoplasts.

Response: Thanks for your suggestions. Although transient transformation of Arabidopsis protoplasts is indeed a powerful approach to recapitulate molecular mechanisms, it is regrettable that TIC, which encodes a large protein of 1,555 amino acids, shows very low transformation efficiency in protoplasts. As a result, using protoplasts derived from the *cca1 lhy* double mutant to express TIC did not yield valid results. In contrast, transformation of CCA1 and LHY into protoplasts prepared from Col-0 and *tic-2* mutant were feasible. Our results showed that when CCA1-HA and LHY-HA were expressed in Col-0 protoplasts, they could significantly repress *PHYA* transcription (Supplemental Fig. 8). However, these transcriptional repression by CCA1 and LHY were significantly attenuated in *tic-2* protoplasts (Supplemental Fig. 8), supporting that TIC may enhance the transcriptional repression of *PHYA* by CCA1/LHY



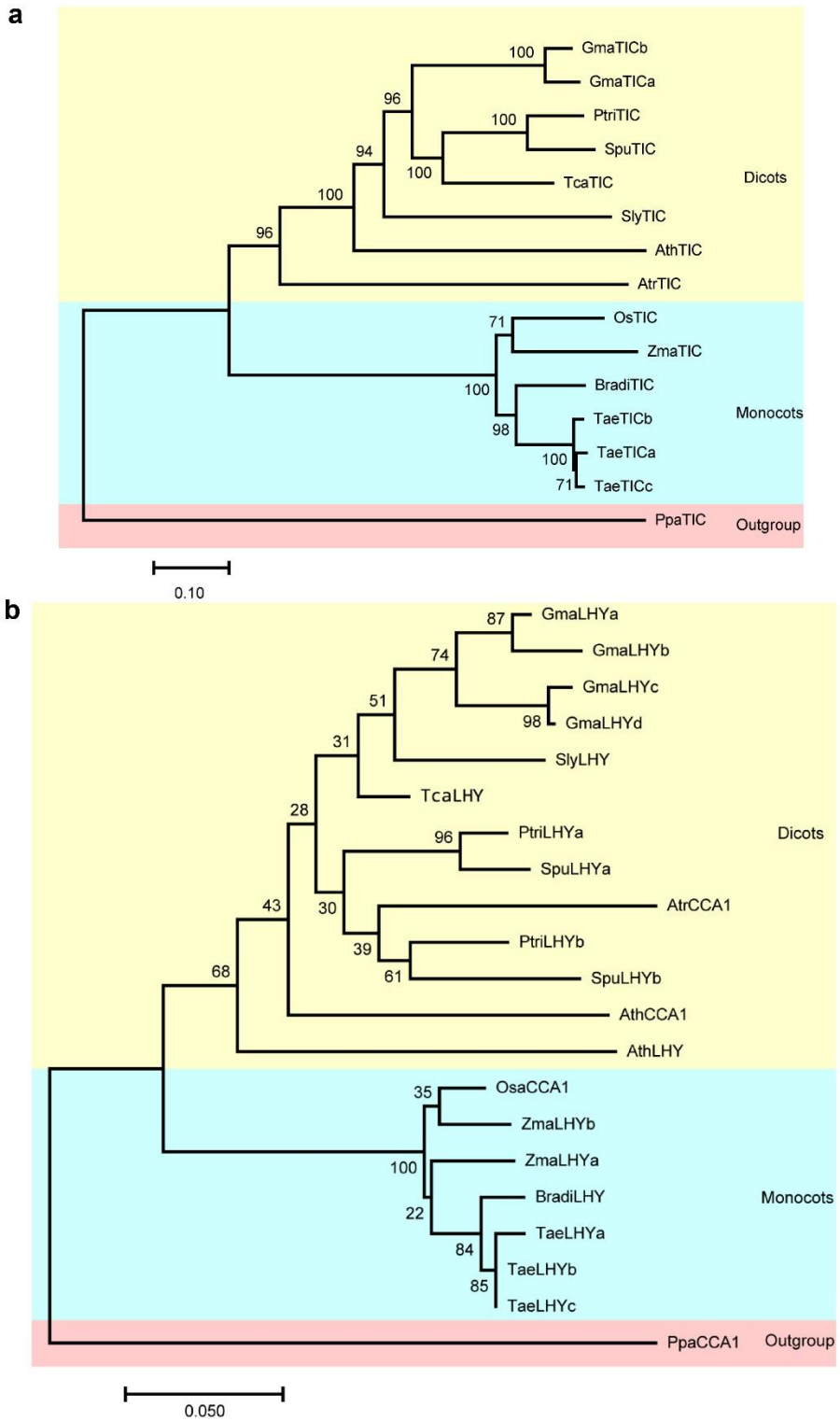
Supplemental Fig 8: TIC may enhance CCA1- and LHY-mediated repression of *PHYA*.

(a) Schematic representation of the effector and reporter constructs used in the protoplast transfection assays.
(b) Relative LUC/REN activities of *PHYAp:LUC* co-transfected with CCA1 or LHY into Col-0 or *tic-2* protoplast, which were normalized to the mean LUC/REN value obtained from co-transformed of *PHYAp:LUC* with 35S:3HA. Data represent mean ±s.d. Asterisks indicate significant differences according to Student's *t*-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).
(c) Immunoblot detecting the respective protein levels in (b).

5. To demonstrate the functional interdependence among MC components, genetic crosses such as TIC-OX in cca1 lhy and CCA1-OX in tic backgrounds would be useful. These could reveal whether one component is required for the other to regulate circadian oscillation and far-red signaling.
Response: Thanks for your suggestion. Although we have already presented the far-red light hypocotyl phenotype of the *cca1 lhy tic* triple mutant to illustrate the genetic relationship of the morning complex in regulating far-red light signaling, the phenotypes of *TIC-OX cca1 lhy* and *CCA1-OX tic* lines could provide further validation. Unfortunately, possibly due to the large size of the TIC protein, no stable *TIC-OX* Arabidopsis lines have been obtained since the beginning of TIC research. Therefore, assessing the phenotype of *TIC-OX cca1 lhy* remains unfeasible for now. As for the *CCA1-OX tic* line, we have not yet obtained a homozygous line due to its extremely late flowering. Nonetheless, it is conceivable that its hypocotyl phenotype under far-red light perhaps resemble that of the *tic-2* mutant, due to hypocotyl phenotype of *cca1 lhy tic* under far-red light closely resembles that of *tic-2*. Besides, the effect of *CCA1-OX* may cause other effects as it can interact with other transcriptional regulators, as the known CDD complex which also interacts with CCA1/LHY[4].

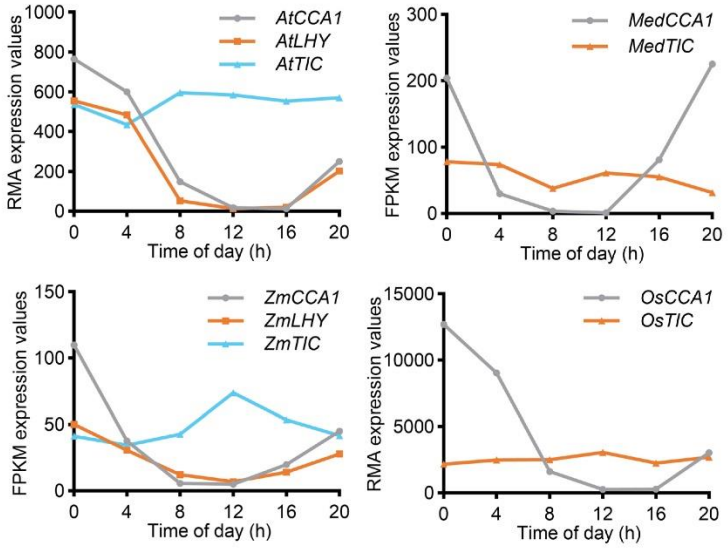
6. While Arabidopsis is an excellent model, a discussion on whether homologs of TIC, CCA1, and LHY co-express or co-function together in other plant species would enhance the broader relevance of the finding. Sequence conservation and promoter motif analysis across species would be helpful.
Response: Thanks for your kind suggestion. We first investigated the evolutionary relationships of TIC, CCA1, and LHY, and the analysis revealed that all three genes originated early in land plant evolution. These genes are highly conserved among angiosperms and exhibit clear divergence between monocot and dicot lineages (Supplemental Fig. 12a and b). We analyzed the diurnal expression patterns of *TIC* and *CCA1/LHY* over a 24-hour cycle in Arabidopsis, alfalfa, maize, and rice. Consistent with findings in Arabidopsis, *CCA1/LHY* exhibited a pronounced peak at dawn in all analyzed species, whereas *TIC* displayed relatively constant expression levels throughout the day (Supplemental Fig. 13). These conserved expression profiles suggest a potential co-expression and functional association between TIC and CCA1/LHY across diverse plant species. Since the functional domains of TIC in Arabidopsis remain unidentified, a detailed analysis of its sequence conservation is currently limited. Nonetheless, studies have shown that TIC participates in the regulation of jasmonic acid (JA)-mediated biotic stress responses in Arabidopsis, and this regulatory role is conserved in monocot species[5,6]. This raises the possibility that TIC's functions in circadian and light signaling pathways may also be evolutionarily conserved. Moreover, numerous reports have demonstrated that the structural domains of CCA1 and LHY are highly conserved across diverse plant species and remain functionally involved in circadian regulation and associated biological processes[7-10]. Collectively, these observations suggest that TIC and CCA1/LHY, which

exhibit co-expression at dawn across species, likely play a conserved role in circadian clock–mediated light signaling pathways.



Supplemental Fig 12: Evolutionary relationships of TIC and CCA1/LHY in green plants. Phylogenetic classification of TIC (a) and CCA1/LHY (b) homologs in green plants, showing their conservation across land plants and a clear evolutionary divergence between monocotyledonous and dicotyledonous species. Gma label indicates *Glycine max*; Ptri label indicates *Populus trichocarpa*; Spu label indicates *Salix purpurea*; Tca label indicates *Theobroma cacao*; Sly label indicates

Solanum lycopersicum; Ath label indicates *Arabidopsis thaliana*; Atr label indicates *Amborella trichopoda*; Os label indicates *Oryza sativa*; Zma label indicates *Zea mays*; Bradi label indicates *Brachypodium distachyon*; Tae label indicates *Triticum aestivum*; Ppa label indicates *Physcomitrella patens*.



Supplemental Fig 13: Co-expression patterns of *CCA1/LHY* and *TIC* homologs in representative plant species.

Co-expression analysis of *CCA1/LHY* and *TIC* were conducted in *Arabidopsis thaliana*, *Medicago sativa*, *Zea mays*, and *Oryza sativa*. The expression data for *Arabidopsis* and *Oryza* were obtained from Diurnal database (http://diurnal.mocklerlab.org/), while data for *Medicago* were sourced from the study, and the expression data for *Zea mays* were retrieved from the paper.

Minor comments

1. Figure 1A, 1D: Please include a negative control using proteins known not to interact with CCA1/LHY in the Co-IP assays to confirm interaction specificity.

Response: Thanks for your valuable suggestion. We added a negative control GFP-TOC1 in Figure 1a to confirm the specificity of CCA1/LHY interaction with TIC. Since the C-terminus of TIC does not interact with CCA1/LHY, it served as the negative control, so Figure 1d was not updated.

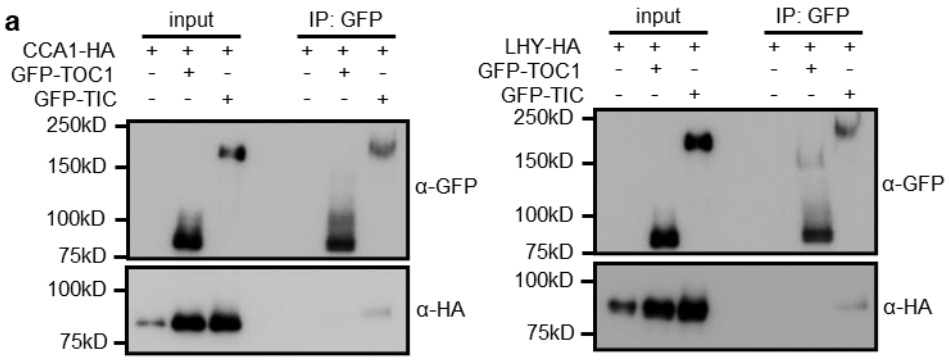


Fig.1a Co-immunoprecipitation analysis showing GFP-TIC interacts with CCA1-HA and LHY-HA in tobacco. No interaction was detected between GFP-TOC1 and CCA1-HA or LHY-HA, serving as a negative control. Total protein extract was generated from transiently co-expressed leaves of

four-week-old *N. benthamiana*, as indicated. Protein A agarose beads with GFP antibody (A11120) were used for immunoprecipitation.

2. Figure 2C: Consider adding functional category annotations to the heatmap for easier interpretation.

Response: Thanks for your helpful suggestion. We agree that adding functional category annotations can enhance the interpretability of the heatmap. Since genes often have multiple functions, in Figure 2c we marked their most common functions—for example, PRR5 and PRR7 as key circadian regulators.

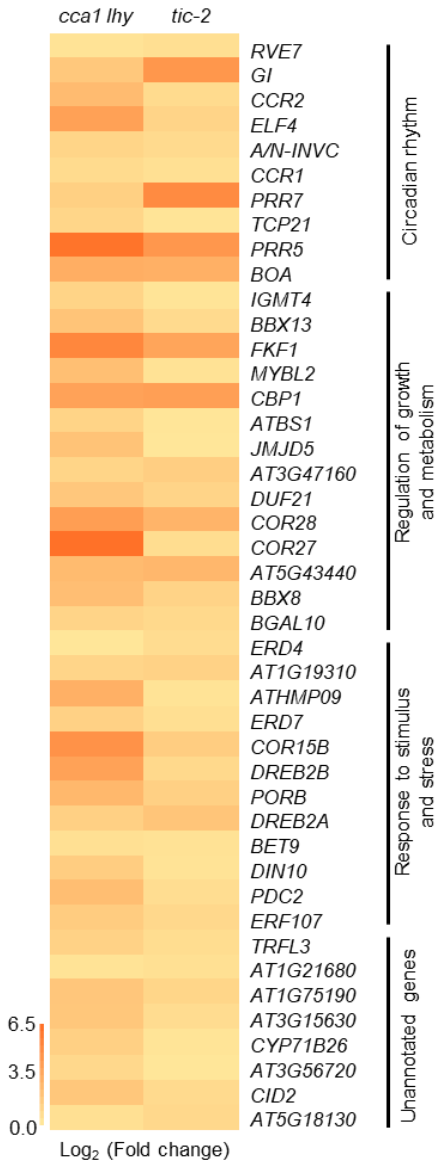


Fig.2c Heat map of 44 overlapping genes in *cca1 lhy* and *tic* mutants. The colored bar represents $\log_2$ (Fold Change).

3. Figure 3B, 3D: Clarify the statistical results corresponding to results.

Response: The data in figures 3b and 3d were analyzed by grouping samples under the same light intensity for statistical comparison. In figure 3b, at each tested fluence rate, the hypocotyls of the mutants were significantly shorter than those of Col-0, while no significant differences were observed among the mutant lines. Similarly, in figure 3d, the overexpression lines showed

significantly longer hypocotyls than Col-0 across each tested FR fluence rate. Slight differences in hypocotyl length were observed among the overexpression lines only at 0.5 μmol $m^{-2}s^{-1}$, whereas no significant differences were detected at the other light intensities.

References:

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7 Lu, S. X., Knowles, S. M., Andronis, C., Ong, M. S. & Tobin, E. M. CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL function synergistically in the circadian clock of Arabidopsis. Plant Physiol 150, 834-843 (2009).

8 Wei, H., Xu, H., Su, C., Wang, X. & Wang, L. Rice CIRCADIAN CLOCK ASSOCIATED1 transcriptionally regulates ABA signaling to confer multiple abiotic stress tolerance. Plant Physiol (2022).

9 Wang, K. et al. Two homologous LHY pairs negatively control soybean drought tolerance by repressing the abscisic acid responses. New Phytol 229, 2660-2675 (2021).

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Reviewer #1 (Remarks to the Author):

I thank the authors for their careful consideration of my comments. While most of my comments have been addressed to my satisfaction, I feel that some further work is required for the following comments.

1. Regarding the response to my comment on the former line 243, I don't agree that the authors' response, alone, resolves this issue without a change in the manuscript.

My comment related to the fact that the compact letter display in Fig 5c does not show a significant difference between Col-0 and the cca1 lhy double mutant at ZT0 for FHL expression as was claimed in the text. (Both receive cld letter b based on a Tukey HSD post-hoc set of pairwise tests according to the legend).

The authors' suggested that this lack of significance was due to "between group differences". While this may be the case, the graph presented still shows no significant difference so the description of the results must reflect this.

If the Tukey's HSD test is not felt to be suitable here, then an alternative statistical text could be applied to the data. The authors mention in their rebuttal that a single pairwise t-test between these two values in isolation shows significance. However, it is not stated whether this involved any correction for multiple testing (e.g. Bonferroni), which would be required as this is just one of many pairwise t-tests that could be applied to that data. If this still shows a significant difference after Bonferroni correction, then it would be valid for the authors to make the claim, but the test would need to be mentioned in the manuscript (and, ideally, the same the test should also be applied to the rest of Fig5c too).

Response: We appreciate your valuable comments and thank you for drawing our attention to the issue of type I error introduced by multiple *t*-tests, which we had inadvertently overlooked in our previous response. Following your suggestion, we applied Bonferroni correction and found that the *p*-value was slightly above 0.05. Therefore, describing this difference as "significant" is not appropriate. Finally, we have not altered the method of significance analysis, and we have revised the wording accordingly in the revised manuscript: "Finally, we examined the transcript levels of *PHYA*, *FHY1*, and *FHL* in *cca1-1 lhy-20 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)."

2. Regarding the response to my comment on the former line 248, I also don't agree that the authors' response fully resolves this issue.

I had mentioned that it looks like the effect of loss of TIC on PRR7 expression at ZT0 is somehow ameliorated by loss of CCA1 and LHY and suggested that this could be discussed. However, I feel that the argument made by the authors in their response - that the regulation of PRR7 by the Morning Complex may be more dependent on CCA1/LHY function - doesn't fully fit the data.

Loss of TIC alone has a much greater impact on PRR7 than either loss of CCA1/LHY alone or loss of all three factors. A possible explanation is that, although CCA1/LHY normally inhibit PRR7 at ZT0, they can also act to positively regulate PRR7 at this time in the absence of TIC. (i.e. TIC changes the sign +/- of the CCA1/LHY effect on PRR7 expression).

Response: Thanks for your critical and helpful suggestion. The mechanism by which CCA1/LHY act as transcriptional repressors or activators remains unclear. Your suggestion is indeed very reasonable and has provided us with valuable insight. Followed your suggestion, we have revised this section as follows in the revised manuscript:
"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 [42], and in rice, OsCCA1 has been reported to activate ABA signaling components through binding to their promoters [43]. 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."

3. Regarding the response to my comment on the former line 269, the authors state that they have removed the phrase "similar to CAB" but this still appears in the amended manuscript. The authors response here that "This [PHYA] expression pattern reflects the outcome of integrated regulatory mechanisms rather than a simple correlation with CCA1/LHY expression dynamics alone" provides a very good alternative explanation to that which appears in the text. It would also be good to include that in the discussion at that point rather than the current text which still just relates PHYA expression level to the action of the morning complex.
Response: Thank you very much for your thoughtful suggestions, and we apologize for the oversight in our previous revision. To avoid overstating the role of the morning complex in regulating *PHYA* transcript levels, we have revised the text as follows into the Discussion part of the revised manuscript:
"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."

Reviewer #2 (Remarks to the Author):
Most of my concerns have been addressed during the revision.
Response: Thank you very much for your insightful and helpful comments to improve our manuscript.