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PSEUDO RESPONSE REGULATORs stabilize CONSTANS protein to promote flowering in response to day length

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Thank you for submitting your manuscript to us.
I have now received the reviews from three referees, which I enclose below.

As you will see, all referees appreciate your study. However, they also think that your conclusions require additional experimental support and further insight to rule out or test alternative explanations and to explain some inconsistencies observed throughout the manuscript.
I would thus like to invite you to provide a revised version of your work. A few critiques raised can be addressed in the text or commented on, but the following points have to be addressed experimentally for further consideration here:

- please provide additional PRR/TOC1 ChIP studies in the co mutant background as outlined by referee #1
- please analyze whether altered translation contributes to the effect on CO stability (referee #2, point 1)
- please strengthen your data on PRR/CO mediated FT expression via promoter binding (referee #2, point 4 and referee #3, point 18)
- please provide a better control for the co-IP in figure 5 (referee #2, point 6)
- please address with further experiments points 3 and 4 of referee #3
- please address point 5, 8, 9, 10, 12, 14, 17 of referee #3
- A more detailed flowering phenotype needs to be described (point 7, referee #3)

Furthermore, the statistical significance of biological replicates needs to be added and the number of biological replicates and the number of samples examined in each replicate need to be stated.

REFEREE REPORTS

Referee #1:

PSEUDO RESPONSE REGULATOR (PRR) proteins control photoperiodic flowering in cereals and beet and in this manuscript authors show that these proteins act cooperatively with Arabidopsis CONSTANS, aiding to CO protein stabilization in the light, in addition to directly activate FT expression by binding a similar promoter region as the CONSTANS factor.
PRRs accumulate during the day and modulate clock function by suppressing LHY and CCA1 transcription. Mutations in the PRR genes delay flowering in LDs, with an overlapping role of these genes in flowering time being thought to be mediated by indirect regulation of CO transcription, due to their effect on the clock. In this work, author's show that PRRs directly interact with CO and mediate morning and evening stabilization of this transcriptional factor, by suppressing COP1capacity to degrade CO. The work shows that this effect is not caused by reduced COP1 function (HY5 is not stabilized), but from specific protection of the CO protein from degradation. FRET and co-IP data are provided showing that PRRs physically interact with CO, co-IP studies with truncated forms of these proteins also showing that interaction requires of the CO CCT domain, reported to be responsible for FT promoter DNA binding and COP1 interaction.
Authors likewise show that FT transcript levels are strongly reduced in prr mutants and elevated in PRR-OX plants, and that FT activation in the over-expresser lines occurs independently of CO mRNA levels. FT transcript levels are in fact reduced in toc1 prr5 prr7 prr9 SUC2::CO lines as compared to SUC2::CO plants, reduced FT expression correlating with a later flowering phenotype, which demonstrates that PRR proteins promote FT transcription independently of transcription of CO. Authors provide ChIP-PCR data showing that PRRs bind the proximal FT promoter region, although binding efficiency was found to be less than for the CCA1 promoter. The PRR-enriched region contains the CORE motifs bound by CO, with evidence for direct PRR and CO interaction hence suggesting that both proteins might bind as a complex the FT promoter and activate transcription of this gene. Together, these data underscore an unexpected novel role for PRRs in photoperiodic flowering, due to stabilize CO during the day and bind the FT promoter possibly in a complex with CO.
These results are novel and very interesting and merit publication since they demonstrate that PRRs convey information on light exposure to CONSTANS and may contribute to explain how PRRs control flowering in crop species where alleles of these genes were identified as main loci for day length recognition. If any, data would be strengthen by additional PRR/TOC1 ChIP studies in the co mutant background, which would further substantiate that FT activation by PRRs requires complex formation with CO, as suggested by the low expression of FT in the co mutant, or reduced FT expression at midday, when PRR7 is expressed.
Minor points:
1. What is the difference between Fig 2A and Fig S2C?
2. Figure S2C should be easier to read if the same color/shapes are used for SUC2::HA:CO in both graphics and a different color for prr79 and toc1prr579. Same for all Figures. Correct tpc1prr57.
3. Figures3I-K and S3F-H are somehow redundant.
4. Is Figure S4 a biological replicate of Figure 4?
5. The pattern of PIF4 transcript accumulation in the toc1prr579 mutant looks odd. Much higher mRNA levels are detected at night compared to daytime and this cannot be explained by impaired evening complex function.
6. In Figure S8 panels A and B are missing.

Referee #2:

Hayama and colleagues present results showing that PRR proteins are important to stabilize CO thereby promoting expression of the florigen gene FT. This data is interesting because regulation of CO levels is of great importance for the transition to reproduction in the model plant Arabidopsis and because the mechanism discovered here may explain how PRR proteins are involved in conferring latitudinal adaptation in flowering time to crop species. This being said some central claims of this work could be strengthened and/or should be clarified.

Major comments

1) The central finding of this paper is that PRR proteins stabilize CO protein. This conclusion relies on the fact that CO protein levels are reduced in prr mutants while CO transcript levels remain relatively unaffected in those mutants. One interpretation is that PRR proteins stabilize CO, however I don't see how other effects such as altering translation could be ruled out. Consistent with the authors hypothesis is the finding that in a cop1prr mutant background CO levels remain high, however this is not really direct evidence for an effect on protein stability either. To address this issue more directly the authors should use proteasome inhibitors to see whether this leads to greater stabilization of CO in prr mutants than in the WT.
2) The authors propose that the temporal order of PRR protein accumulation explains stabilization of CO at different times of the day. The data clearly supports an additive effect on CO protein levels when combining different prr mutants (e.g. Figure 3). Moreover, it is true that CO accumulation at ZT1 is quite normal in toc1prr57, while it is clearly affected in prr9 (Figure 3I). However, the importance of this temporal order and the reproducibility of this CO accumulation data is a bit questionable when one compares data from Figure 3I (4X difference according to quantification in 3J) with Fig S3G where the prr9 effect appears to be barely 2X. The authors also argue that this morning accumulation of CO leading to FT expression is important for the regulation of flowering time. If this were true then one would expect an obvious flowering phenotype in prr9 mutants. Is this really the case? If the authors want to insist on the importance of this temporal order of action of the different PRRs on CO accumulation, they should provide some better evidence. Alternatively, they should tune this down in the text.
3) There appears to be a bit of a disconnection between the very large effect of the prr mutants on FT expression and their effect on flowering time. For example both prr7prr9 and prr5prr7 have huge effects on FT expression (Figure 1B) while it is only in the toc1prr579 quadruple mutant that obvious effects on flowering time are observed (Figure 2G). Unfortunately the flowering time phenotype of prr7prr9 and prr5prr7 is not shown. The authors should clarify this.
4) The authors propose that PRRs bind to the FT promoter together with CO and thereby promote FT expression. This data is not very convincing (very modest enrichment of PRR on Figure 6). Was such binding of PRR on the FT promoter also observed in several genome-wide PRR ChIP seq studies that were recently performed (e.g. recently published Liu et al., 2016 in Plant Phys, that should also be cited here)? Moreover the functional consequences proposed by the authors of such a common binding (page 22 of the discussion) could easily be tested. The authors have SUC2::HA:CO in cop1 and in cop1toc1prr579 quintuple mutants (Figure 4). In both cases CO levels are high. What happens to flowering time and FT expression in those mutant backgrounds? If PRRs are directly important for FT expression beyond a stabilizing effect on CO, FT expression and flowering are expected to be delayed in cop1toc1prr579 compared to cop1. This experiment should be rather quick to perform due to early flowering in cop1.
5) The authors should comment on the discrepancy between the effect of FKF1 on CO protein accumulation reported in Song et al., 2012 (Science) compared to the data presented here in figure 3. Could this be because in Song et al., CO was driven by the 35S promoter while here it is driven by the SUC2 promoter?
6) PRR-CO interaction on figure 5. The control used in panels C and D should not be an empty vector but GFP. The data presented here does not allow discriminating between an interaction with PRRs or GFP. It is very difficult to see anything for the PRR:CFP images in panel A. Could the authors present images that are more clear?
7) For all the figures from the paper it should clearly be stated what exactly is being shown for the quantified gene expression values and protein level values. This is not always clear from all the figure legends. Do the authors show averages from biological repeats or averages from technical repeats of a representative experiment? N=? errors shown as SD or SE?
8) Also regarding gene expression data. Gene expression is typically shown as relative data (not absolute number of transcript which is very hard to get). For the panels (most of them) when data for multiple genotypes are shown what exactly do the authors show? Expression relative to one time point in one genetic background? Please make this clear in materials and methods and/or in the legend of the first figure showing such data.

Minor comments

1) A map of the CO fragments used in panel 5D would be useful

2) Review bibliography for some inconsistencies
3) The text often refers to significant differences between genotypes but this is not tested statistically, please clarify.

Referee #3:

The manuscript by Hayama et al., focuses on the role of PRRs controlling CONSTANS protein stability under long day conditions. The authors use a series of mutants to examine gene and protein accumulation under long days and particular light conditions. The authors also use FRET and protein co-immunoprecipitation in transient assays to demonstrate the interaction between the PRRs and CO. Furthermore, chromatin immunoprecipitation assays reveal that PRRs bind to the FT promoter. As stated, the main conclusion of the manuscript is that: "the diversity in the timing of expression of the PRRs allows CO to accumulate at specific times during the day to generate the typical LD-specific accumulation pattern of CO".

The general topic of the manuscript is highly relevant and the main claim, if fully demonstrated, is significant and sound. However, due to the complexity of the PRR and flowering regulatory networks, some of the conclusions are arguable and require additional experiments while other parts of the data do not fully support the author´s claims and present some inconsistencies that need to be further clarified. More specifically:

1.
In Figure 1, the authors show the uncoupling of CO and FT mRNA accumulation in plants mis-expressing PRRs. The uncoupling effect is clear on Figure 1C and D but not so much in A and B, as the reduced expression of CO in prr79 or prr57 before dusk correlates quite well with the reduced expression of FT.

2.
It is also worth noting the up-regulation of CO at dawn in prr79 as compared to the down-regulation of prr57 at this time point. Therefore, the results indicate that the PRRs have a very important role regulating the transcription of CO. This notion should be taken into consideration when drawing conclusions about the role of PRRs regulating photoperiod through CO function. Also, based on the sequential regulation among the PRRs, it would be important to check the expression of all the PRRs in the mutants and over-expressing lines (e.g. the expression of other PRRs will be affected in PRR5-ox lines).

3.
In Figure 2, the authors use SUC2::CO lines to express CO in phloem companion cells and to avoid the interference of an impaired clock. However, these lines might not be quite appropriate for these purposes as the expression of SUC2 rhythmically oscillates and clock function in veins was reported to be very important.

4.
Consistent with these concerns, panel 2A shows a somehow oscillating pattern of CO mRNA but this pattern is not coinciding with the one described for SUC2. Any idea why? Also, are the SUC2::CO lines in a WT background or in co mutant background?

5.
It is also intriguing that FT expression in toc1prr5 is almost half of that observed in WT plants (Figure 2F) but this reduced expression does not lead to a flowering phenotype (Figure 2G).

6.
PRR3 is arguably expressed in veins. Did the author check the effect of PRR3 mis-expression on CO stability in phloem companion cells?

7.
Flowering phenotypes are presented as changes in the number of leaves. Do the authors reach the

same conclusions by checking the alteration in the number of days to flowering?

8.
In Figure 3, the authors describe changes in CO protein accumulation on several prr mutants. The authors conclude that the sequential wave of PRR expression regulates CO stability at different phases during the light period of long-day cycles. This is an interesting idea and the conclusion seems to be supported by Figure 3J. However, the pattern of CO accumulation (Figure 3F) shows that there is almost no CO protein around 4-8 hours, i.e. at the time of PRR9, 7 and 5 function. It is then difficult to reconcile a major function for PRRs stabilizing CO protein at a time when there is almost no CO protein. This could be due to differences between the whole plant and phloem companion cells but as the raising phase of CO seems to be advanced in SUC2::HA:CO, the waveforms could be due to clock impairment in the veins of the prr mutants (please see below the comments to Figure S6).

9.
Also, if PRRs sequentially control the timing of CO stability as shown in Figure 3J, then single mutants should present some phenotypes, which seems not to be the case (e.g. SUC2::HA:CO prr9 shows reduced CO accumulation in the morning but this is not reflected in changes on FT expression (Figure 2D) or in flowering phenotype (Figure 2G).

10.
The authors also show that the fkf1 mutation did not strongly affect CO accumulation. This is in contrast to previous observations but also it is not consistent with the fact that the ZTL protein family controls the stability of PRRs. Are the authors implying that PRR protein degradation is not regulated by the ZTL family in phloem companion cells?

11.
In Figure 4, the authors focus on the light-dependent function of PRRs on CO protein stabilization. The authors perform analyses under BL and FRL. So, under Red Light (RL), regulation is just transcriptional? Are the PRRs not functioning? This is intriguing as the function of some PRRs (e.g. on hypocotyl elongation) is particularly evident under RL. It would be interesting to know whether CO protein is stabilized in PRR-ox lines under RL.

12.
In Figure 4F, there is detectable protein and a clear oscillation of CO protein in toc1prr579 (red dotted line). However, in Figure 3J, CO protein is quite low throughout the whole cycle (red line). What are the reasons for such discrepancy?

13.
The authors conclude that PRR proteins contribute to light-mediated accumulation of CO. In Figure 4F, it seems that in cop1 toc1prr579 there is less CO protein also during the night (ZT20). Is this reproducible? Are the differences significant among the biological replicates?

14.
The authors also compare cop1 with cop1toc1prr579 mutant lines to conclude that PRRs stabilize CO by suppressing the capacity of COP1 to degrade CO. The results indicate that the prr phenotype requires the presence of a functional COP1. However, full demonstration that PRRs suppress COP1 function would require additional functional evidence. For instance, did the authors check whether expression of COP1 is affected in the prr mutants? Also, the authors could check CO stability in COP1-ox lines (or inducible ox) and then examine whether over-expression of PRRs enhances the stability of CO. As the main topic of the manuscript relates to PRRs and stability of CO protein, including these experiments might be a bit out of the scope of the manuscript, but if the authors maintain their statement in results and discussion, then compelling evidence showing that PRRs suppress COP1 activity is necessary.

15.
The authors also checked hypocotyl elongation and whether other COP1 targets (such as HY5) might be also affected in the prr mutants. Quite surprisingly, they found that the long hypocotyl phenotype in toc1prr5prr7prr9 was observed only under LDs and SDs but not under continuous light. This is difficult to reconcile with previous reports showing for instance a clear hyposensitive

phenotype in several prr mutants under RL [e.g. Nakamichi et al., Plant Cell Physiol. 46(5): 686-698 (2005)].

16.
The pattern of PIF4 expression in toc1prr5prr7prr9 (Figure S5E) is practically the same to that shown for CO in Figure 2A. Is this a mistake?

17.
Figure 5 shows data about the physical interaction between PRRs and CO. These results are interesting but there are a number of questions and issues that need to be addressed:

- At what time was FRET performed? Are there changes in the interaction depending on the time-of-day?
- If light stabilizes CO protein trough PRR function: Do you see interaction in the dark?
- Some PRR proteins (e.g. TOC1) are localized in well-defined nuclear speckles. These speckles are not clearly visible in Figure 5A.
- Why CO-YFP localization is so different in the four panels of Figure 5A?
- If PRRs stabilize CO protein, then its accumulation should be overall higher in double PRR/CO compared to single CO transformation. Do you see that?
- The Y axis in Figure 5B is labeled as "FRET Efficiency (%)". Does this efficiency reflect the percentage of donor decreased fluorescence before and after acceptor bleaching?
- Figure 5C shows that the interaction of CO with PRR9 is clearly lower than with the other PRRs (at least based on the amount of immunoprecipitated proteins). Was this result consistently observed in the different biological replicates? The FRET analyses suggest otherwise.
- It is a pity that the authors cannot examine the interaction using stable transgenic Arabidopsis lines because the results using these plants could provide conclusive information about the timing and the possible light-dependent interaction.

18.
In Figure 6 the authors show that PRRs bind to the FT promoter. However, this figure is not properly explained, which complicates the interpretation of the results. For instance, are the numbers 1-4 on the X axis corresponding to sampling at ZT1, 8, 12 and 16? It is not clear in the figure legend. Are the authors expecting a differential binding depending on the time of PRR function? It doesn´t seem the case. The manuscript´s conclusions would be also clearly reinforced if the authors compare the binding of CO to the FT promoter in HA:CO versus HA:CO/PRRs. If PRRs stabilize CO protein, binding could be also enhanced.

19.
In Figure S6, the authors attempt to demonstrate that the phenotypes are independent from the clock. However, conceptually, this idea is a bit incoherent with the main conclusion that the timing of PRR function regulates CO stability at different phases during the light period of long-day cycles. Indeed, timing and phase of PRRs are intimately ligated to the clock function. In any case, methodologically, just checking FKF1 or TOE1 expression is not compelling enough to conclude that the phenotypes are independent from the clock. Also, they refer to the prr9 mutant, but this mutant do not show changes on FT expression (Figure 2D) or in flowering (Figure 2G). If the authors want to examine independence from the clock, they could "eliminate" timing for instance by growing the seeds for several days under continuous light (without any previous synchronization).

20.
The Discussion is interesting but as mentioned in the comments of Figure 4, the authors should either provide compelling evidence that PRRs stabilize CO by suppressing the capacity of COP1 to degrade CO or decrease the tone of their conclusions accordingly with the actual results that they provide. Also, the authors should include in Discussion (and/or Introduction) the recent findings by the Imaizumi´s lab: Distinct roles of FKF1, Gigantea, and Zeitlupe proteins in the regulation of Constans stability in Arabidopsis photoperiodic flowering. Song YH, Estrada DA, Johnson RS, Kim SK, Lee SY, MacCoss MJ, Imaizumi T. (Proc Natl Acad Sci U S A. 2014 Dec 9;111(49):17672-7).

Other comments:

1. The results should be accompanied by statistical significance of biological replicates. The number

of biological replicates and the number of samples examined in each replicate should be clearly stated.
2. Many panels in Supplemental Figures are not described or even mentioned in the text (e.g. Figure S2B, S5C, S6D, E, F, etc).
3. Please revise grammar and typos throughout the manuscript.
4. Figure 1 mostly refers to the uncoupling between CO mRNA and FT mRNA. Based on previous reports about the importance of CO protein stability, the novelty of this figure is relatively minor in the context of the whole manuscript. Therefore, if needed, this figure could go to supplemental.
5. In the Introduction (page 4) when it says: "This coincidence between CO protein and light exposure allows stabilization of the protein, causing CO to accumulate under LDs and achieving recognition of LDs (Fig. S1) (Valverde et al., 2004)" it should say: "This coincidence between CO mRNA..., right?
6. The authors state several times in the manuscript that the components and mechanisms from light to flowering "remain unclear". This statement is a bit misleading. The myriad of components and mechanisms thus far described are clear, maybe it would be more accurate to say that they are incomplete (or similar).
7. In Materials and Methods, the light and temperature conditions in which the plants were grown in the different experiments should be specified.

1st Revision - authors' response 07 December 2016

Response to the reviewers

We have extensively revised the text in response to the reviewers' comments and have included important new data. Most of these data strengthen our previous conclusions. In the new experiments ChIP of HA:CO to the *FT* promoter was performed, as suggested by reviewer 3. This experiment demonstrates that in the *prr* quadruple mutant CO binding to *FT* is indeed reduced compared to WT, consistent with the lower abundance of CO protein and explaining the reduction in *FT* mRNA observed. Also CO binding is increased in TOC1 overexpressor plants. However, in the revised version the ChIP of PRRs to *FT* promoter has been deleted. The enrichment previously detected was weak, as pointed out by more than one reviewer, and could not be reproduced. The precise changes made are described in more detail below in response to the specific points raised by the editor and reviewers.

Editor's comment:
- please provide additional PRR/TOC1 ChIP studies in the *co* mutant background as outlined by referee #1
Response: We have carried out extensive additional ChIP experiments to test binding of PRRs to *FT* in wild-type and *co* mutant background. However, in these experiments we could not reproduce the enrichment of *FT* promoter after co-immunoprecipitation of PRRs detected in the original ChIP data. Therefore, in this revised version we have removed these ChIP data from the manuscript.

Instead, we have included a new ChIP experiment in which we analyze HA:CO binding to *FT*. This experiment shows decreased enrichment of the *FT*-promoter segments to which HA:CO binds in the *prr* quadruple mutant compared to WT plants (Fig. 6). This result demonstrates a functional connection between CO stabilization through PRRs and induction of *FT* transcription and flowering. Furthermore, ChIP data were added showing that enrichment of the *FT*-promoter segments through HA:CO are enhanced in a *TOC1* overexpressor (Fig. S8). We have included this information on pages 12-13 in the Results and in the Discussion on page 14.

We have also deleted statements related to direct transcriptional roles of PRRs on *FT*, focusing instead on their roles in stabilizing CO during the day allowing it to accumulate under LDs and directly promote *FT* transcription. These new data are fully consistent with the major argument of our paper that PRR proteins have a role in the stabilization of CO.

- please analyze whether altered translation contributes to the effect on CO stability (referee #2, point 1)

Response: In the revised version of the manuscript we have tested the stabilization of HA:CO in *SUC2*::*HA:CO* and *toc1prr5prr7prr9 SUC2*::*HA:CO* plants after addition of MG132, which blocks activity of the proteasome. We compared the fold increase in HA:CO accumulation in response to

MG132 treatment *SUC2*::*HA:CO* and *toc1prr5prr7prr9 SUC2*::*HA:CO* plants (Fig. S3). HA:CO accumulated to a greater extent upon treatment of *toc1prr5prr7prr9 SUC2*::*HA:CO* plants with MG132 than on treatment of WT plants. This result is consistent with our interpretation that the reduced level of HA:CO in *toc1prr5prr7prr9 SUC2*::*HA:CO* plants occurs through proteasome-mediated degradation rather than by other mechanisms such as reducing CO translation. We have included this information on page 9 in the Results.

- please strengthen your data on PRR/CO mediated FT expression via promoter binding (referee #2, point 4 and referee #3, point 18)
Response: As discussed above, we now demonstrate that *toc1prr5prr7prr9* and *35S:TOC1* respectively reduce and increase CO binding to the *FT* promoter (Fig. 6, S8 and on pages 12-13 in the Results). This presumably has a direct effect on *FT* transcription explaining the differences in *FT* mRNA observed.

- please provide a better control for the co-IP in figure 5 (referee #2, point 6)
Response: In this new version we include data of co-IP with TRB3 as the negative control. TRB3 is a nuclear protein and is ideal for these experiments, because like PRR it is retained in the nucleus during the nuclear extraction protocol used to extract proteins prior to co-immunoprecipitation. In this experiment we used TRB3 protein fused to YFP, a close derivative of GFP, showing that HA:CO is co-immunoprecipitated with TOC1:GFP but not with TRB:YFP (Fig. S7 and on page 12 in the Results). GFP and YFP only differ at a single amino acid that affects absorbance and emission spectra (1), are recognized by the same antibody and were used interchangeably in this experiment.

(1) Rizzo, M.A., Davidson, M.W. and Piston, D.W. (2009) Fluorescent protein tracking and detection: Fluorescent protein structure and color variants. *Cold Spring Harb Protoc*, ***4***, 1-21

- please address with further experiments points 3 and 4 of referee #3
Response: We have included data showing the expression pattern of *CO* mRNA driven by the *SUC2* promoter in LL, and of the *SUC2* gene under LD (Fig. S9). As the reviewer indicates the transcript levels of *SUC2* have been demonstrated to show a circadian rhythm (Michael J. Haydon et al., 2011), whereas we could not detect a strong effect of impaired circadian clock function in the *prr* mutants on the expression of *CO* mRNA when driven by the *SUC2* promoter. Before using the lines that carry *SUC2*::*CO* the expression of *SUC2* mRNA was tested in constant light followng LDs, and we found that the levels of the *CO* transcript do not oscillate, as shown in these data. However, our data do show that *CO* mRNA level in *SUC2:CO* plants is increased during the night and reduced immediately after exposure to light, so we suppose that in the light-dark cycles used here transcript levels of *CO* driven by the *SUC2* promoter mainly respond directly to light and dark, and are only weakly affected by impaired clock function in *prr* mutants. We have briefly explained this information in the supplementary figure 10 legend on page 33 of the manuscript and cite this in the Discussion on page 14 in the main text.
We previously introduced *prr* mutations with T-DNA into a *35S*::*CO* line, but in these lines the 35S::*CO* transgene was silenced, presumably due to interaction with the *35S* promoters present in T-DNAs inserted in the *prr* mutant alleles. Therefore *SUC2*::*CO* and SUC2::*HA:CO* lines provide more reliable information both because this promoter limits expression of *CO* and *HA:CO* to phloem companion cells where endogenous *CO* functions and because it is insensitive to cosuppression causes by the T-DNAs inserted in the *prr* mutant alleles.

- please address point 5, 8, 9, 10, 12, 14, 17 of referee #3
Response: We have addressed these points in the detailed responses to reviewer 3 below.

- A more detailed flowering phenotype needs to be described (point 7, referee #3)
Response: In the Supplementary data section in the revised manuscript we provide flowering-time data showing the number of days to flowering of relevant genotypes (Fig. S2 and page 7 in the Results).

- Furthermore, the statistical significance of biological replicates needs to be added and the number of biological replicates and the number of samples examined in each replicate need to be stated.

Response: In the revised version we have combined biological replicates of expression data and shown statistical significance. For all experiments the number of biological replicates used are stated in the legend. The only exceptions to the use of biological replicates in the main figures are the data in Fig 3I and K where samples early in the morning were harvested at different times (ZT0.5 and ZT1). We put both of these data in the main figure section in the new version (Fig. 3I, J, K, L). The number of seedlings used for RNA and protein expression analyses is now shown in the method section (page 18 in the text). As for flowering time and FRET data, we did not combine the replicates since among the replicates we measured flowering time using different growth chambers. The value of FRET efficiency also varies among performance of replicates, probably due to technical issues in using the microscope, although the observed trends among these results do not vary. We have also added a second biological replicate for the flowering- time data (Fig. 2 and Fig. S2) and FRET data (Fig. 5 and Fig. S7). The new ChIP data are provided with error bars within biological replicates (Fig. 6 and Fig. S8).

Referee #1:
PSEUDO RESPONSE REGULATOR (PRR) proteins control photoperiodic flowering in cereals and beet and in this manuscript authors show that these proteins act cooperatively with Arabidopsis CONSTANS, aiding to CO protein stabilization in the light, in addition to directly activate FT expression by binding a similar promoter region as the CONSTANS factor.
PRRs accumulate during the day and modulate clock function by suppressing LHY and CCA1 transcription. Mutations in the PRR genes delay flowering in LDs, with an overlapping role of these genes in flowering time being thought to be mediated by indirect regulation of CO transcription, due to their effect on the clock. In this work, author's show that PRRs directly interact with CO and mediate morning and evening stabilization of this transcriptional factor, by suppressing COP1capacity to degrade CO. The work shows that this effect is not caused by reduced COP1 function (HY5 is not stabilized), but from specific protection of the CO protein from degradation. FRET and co-IP data are provided showing that PRRs physically interact with CO, co-IP studies with truncated forms of these proteins also showing that interaction requires of the CO CCT domain, reported to be responsible for FT promoter DNA binding and COP1 interaction.
Authors likewise show that FT transcript levels are strongly reduced in prr mutants and elevated in PRR-OX plants, and that FT activation in the over-expresser lines occurs independently of CO mRNA levels. FT transcript levels are in fact reduced in toc1 prr5 prr7 prr9 SUC2::CO lines as compared to SUC2::CO plants, reduced FT expression correlating with a later flowering phenotype, which demonstrates that PRR proteins promote FT transcription independently of transcription of CO. Authors provide ChIP-PCR data showing that PRRs bind the proximal FT promoter region, although binding efficiency was found to be less than for the CCA1 promoter. The PRR-enriched region contains the CORE motifs bound by CO, with evidence for direct PRR and CO interaction hence suggesting that both proteins might bind as a complex the FT promoter and activate transcription of this gene. Together, these data underscore an unexpected novel role for PRRs in photoperiodic flowering, due to stabilize CO during the day and bind the FT promoter possibly in a complex with CO.
These results are novel and very interesting and merit publication since they demonstrate that PRRs convey information on light exposure to CONSTANS and may contribute to explain how PRRs control flowering in crop species where alleles of these genes were identified as main loci for day length recognition.

Response: We appreciate the reviewer's interest in the manuscript and our data, particularly in the capacity of the PRRs to stabilize CO.

Major point:
If any, data would be strengthen by additional PRR/TOC1 ChIP studies in the co mutant background, which would further substantiate that FT activation by PRRs requires complex formation with CO, as suggested by the low expression of FT in the co mutant, or reduced FT expression at midday, when PRR7 is expressed.

Response: The major point raised by the reviewer concerns the ChIP of PRRs to the *FT* promoter and whether this depends on CO. As described above, we performed several additional ChIP experiments to test whether CO affects the binding efficiency of PRRs to the *FT* promoter. However, while carrying out these experiments, we could not reliably reproduce the original ChIP

data and currently we cannot conclude with certainty that PRRs bind to the *FT* promoter. However, we have added a different ChIP experiment that builds on the previous demonstration of CO binding to *FT* promoter and our data that CO protein stability is reduced in *prr* quadruple mutants. We now show decreased enrichment of the *FT*-promoter segments by HA:CO in the *prr* quadruple mutant (Fig. 6). This result provides a functional connection between CO stabilization through PRRs and induction of *FT* transcription and of flowering. We also added a further ChIP experiment showing that enrichment of the *FT*-promoter segments through HA:CO is enhanced in a *TOC1* overexpressor (Fig. S8). We have also described this new information on pages 12-13 in the Results and in the Discussion on page 14.

Minor points raised by the reviewer:
1. What is the difference between Fig 2A and Fig S2C?
Response: These are flowering-time data for *SUC2*::*CO* and *SUC2*::*HA:CO* backgrounds, respectively. They demonstrate the flowering time of individual transgenic lines and that the HA:CO fusion protein is functional.

2. Figure S2C should be easier to read if the same color/shapes are used for SUC2::HA:CO in both graphics and a different color for prr79 and toc1prr579. Same for all Figures. Correct tpc1prr57.
Response: According to the suggestion of the reviewer, we changed the color of all of the corresponding data in the revised version.

3. Figures3I-K and S3F-H are somehow redundant.
Response: These experiments show time-dependent effects of *prr* mutations on HA:CO protein accumulation and are critical for our conclusions. They differ slightly, because the morning samples were harvested at different times (1 h and 0.5 h after light on for the previous main and supplementary figure version, respectively). In the revised manuscript, we included both of these experiments in the main figure to show that the same trend can be observed among genotypes at these times (Fig. 3I, J, K, L). In the supplementary figure section we also included an additional experiment that also shows that in the *SUC2*::*HA:CO prr9* mutant HA:CO accumulation is reduced early in the morning (Fig. S4). We believe that showing these data is important to convincingly support our argument that different PRRs contribute to CO stabilization at different times.

4. Is Figure S4 a biological replicate of Figure 4?
Response: Yes, these figures show biological replicates. In the revised version we combined three data sets to show one graph with error bars in the new Fig. 4B.

5. The pattern of PIF4 transcript accumulation in the toc1prr579 mutant looks odd. Much higher mRNA levels are detected at night compared to daytime and this cannot be explained by impaired evening complex function.
Response: We have not studied this in detail, because it is outside the scope of this paper. However, the evening complex that is composed of the clock components ELF3, ELF4, and LUX was proposed to suppress *PIF4* expression early in the night. Based on this model we suppose that in the *prr* quadruple mutant activity of this clock-controlled complex may be reduced, leading to higher *PIF4* mRNA accumulation during the night (Figure S5B). However, it remains possible that the higher accumulation of *PIF4* mRNA during the night might also be caused by an independent dark-mediated mechanism whose effect only appears under the impaired clock conditions found in the *prr* mutant.

Independently from our *PIF4* expression data in the *prr* quadruple mutant, an increase in *PIF4* transcripts in a *prr* triple mutant has been previously reported (Nakamichi et al., 2009). In this paper they performed microarray analyses to compare gene expression between WT and *prr5 prr7 prr9*, and found *PIF4* among the genes whose transcript levels were up-regulated in this mutant background. We have cited this paper as support for our observation.

Referee #2:

Hayama and colleagues present results showing that PRR proteins are important to stabilize CO thereby promoting expression of the florigen gene FT. This data is interesting because regulation of CO levels is of great importance for the transition to reproduction in the model plant Arabidopsis

and because the mechanism discovered here may explain how PRR proteins are involved in conferring latitudinal adaptation in flowering time to crop species. This being said some central claims of this work could be strengthened and/or should be clarified.

Response: We appreciate the reviewer's interest in our work and suggestions for strengthening the data.

Major comments:

1) The central finding of this paper is that PRR proteins stabilize CO protein. This conclusion relies on the fact that CO protein levels are reduced in prr mutants while CO transcript levels remain relatively unaffected in those mutants. One interpretation is that PRR proteins stabilize CO, however I don't see how other effects such as altering translation could be ruled out. Consistent with the authors hypothesis is the finding that in a cop1prr mutant background CO levels remain high, however this is not really direct evidence for an effect on protein stability either. To address this issue more directly the authors should use proteasome inhibitors to see whether this leads to greater stabilization of CO in prr mutants than in the WT.
Response: We performed the suggested experiment by monitoring the amount of HA:CO protein in *SUC2*::*HA:CO* and *toc1prr5prr7prr9 SUC2*::*HA:CO* seedlings before and after treating with MG132. We found that in the latter genotype MG132 leads to greater stabilization of HA:CO protein. These data are consistent with our proposal that *prr* mutations increase stability of HA:CO by inhibiting proteasome-mediated degradation rather than reducing translation. We provide these data in the new supplementary figure section (Fig. S3) with the related description on page 9 in the Results.

2) The authors propose that the temporal order of PRR protein accumulation explains stabilization of CO at different times of the day. The data clearly supports an additive effect on CO protein levels when combining different prr mutants (e.g. Figure 3). Moreover, it is true that CO accumulation at ZT1 is quite normal in toc1prr57, while it is clearly affected in prr9 (Figure 3I). However, the importance of this temporal order and the reproducibility of this CO accumulation data is a bit questionable when one compares data from Figure 3I (4X difference according to quantification in 3J) with Fig S3G where the prr9 effect appears to be barely 2X. The authors also argue that this morning accumulation of CO leading to FT expression is important for the regulation of flowering time. If this were true then one would expect an obvious flowering phenotype in prr9 mutants. Is this really the case? If the authors want to insist on the importance of this temporal order of action of the different PRRs on CO accumulation, they should provide some better evidence. Alternatively, they should tune this down in the text.
Response: The reviewer is correct to point out that the effect of the *prr9* mutation on HA:CO accumulation early in the morning is variable but we are confident of this result and have included additional supporting data in the new version. We have also specifically mentioned the variability in the effect of *prr9* in the Discussion section on page 14 of the new version. The morning peak of HA:CO accumulation tends to become greater if the plants are grown more densely on a plate, so slight differences in density among experiments or related effects such as shading might have contributed to the variability. We have included two independent experiments showing a reduction in the morning in HA:CO level in *prr9* in the main figure section (Fig. 3), while adding in the supplementary figure section another data set that confirms reduction of HA:CO level in the *SUC2*::*HA:CO prr9* mutant (Fig. S4A). We also add data that show reduced *FT* mRNA levels in the same plant samples (Fig. S4B). A *prr9* mutant was previously shown to flower slightly late (Nakamichi et al., 2005), and we cite this observation on p.14. However, as the reviewer mentions, *SUC2*::*HA:CO prr9* does not generally exhibit late flowering, possibly because high HA:CO levels at other times of the day are sufficient to promote very early flowering of this genotype. Also, *SUC2*::*HA:CO toc1prr5prr7prr9* shows later flowering than *SUC2*::*HA:CO toc1prr5prr7*, showing the effect of the *prr9* mutation on flowering time in the *CO* overexpressor and the redundancy among PRRs.

3) There appears to be a bit of a disconnection between the very large effect of the prr mutants on FT expression and their effect on flowering time. For example both prr7prr9 and prr5prr7 have huge effects on FT expression (Figure 1B) while it is only in the toc1prr579 quadruple

mutant that obvious effects on flowering time are observed (Figure 2G). Unfortunately the flowering time phenotype of prr7prr9 and prr5prr7 is not shown. The authors should clarify this.
Response: In figure 1 we used *prr7prr9* and *prr5prr7* in the Col background where *CO* is expressed from the endogenous gene, whereas in figure 2 we used *toc1prr5prr7prr9* in the *SUC2*::*CO* background where CO levels are increased. The drastic effects on *FT* in *prr7prr9* and *prr5prr7* in Figure 1 are observed due to dual effect of the *prr* mutations on both *CO* mRNA and CO protein accumulation, whereas in the *SUC2*::*CO* background the effects on *CO* mRNA accumulation are absent and to see a strong effect on flowering time through reducing only CO protein stabilization it is necessary to incorporate the four mutations *toc1prr579.* The effect of redundancy among PRRs becomes obvious especially in *SUC2*::*CO*, because *CO* is overexpressed and the transcript level is almost independent of the PRRs. The flowering times of *prr* mutants in Col background have been published previously, for example by Nakamichi et al., (2005, 2007) and are cited.

4) The authors propose that PRRs bind to the FT promoter together with CO and thereby promote FT expression. This data is not very convincing (very modest enrichment of PRR on Figure 6). Was such binding of PRR on the FT promoter also observed in several genome-wide PRR ChIP seq studies that were recently performed (e.g. recently published Liu et al., 2016 in Plant Phys, that should also be cited here)? Moreover the functional consequences proposed by the authors of such a common binding (page 22 of the discussion) could easily be tested. The authors have SUC2::HA:CO in cop1 and in cop1toc1prr579 quintuple mutants (Figure 4). In both cases CO levels are high. What happens to flowering time and FT expression in those mutant backgrounds? If PRRs are directly important for FT expression beyond a stabilizing effect on CO, FT expression and flowering are expected to be delayed in cop1toc1prr579 compared to cop1. This experiment should be rather quick to perform due to early flowering in cop1.
Response: The reviewer is correct that the level of enrichment of the *FT* promoter by PRR ChIP was low although previously we repeated this several times. However, during the revision process we could not repeat these results and have therefore removed them from the figures. Now we show ChIP of HA:CO instead and do not argue for direct binding of PRR protein to the *FT* promoter.

5) The authors should comment on the discrepancy between the effect of FKF1 on CO protein accumulation reported in Song et al., 2012 (Science) compared to the data presented here in figure 3. Could this be because in Song et al., CO was driven by the 35S promoter while here it is driven by the SUC2 promoter?
Response: We suspect that in the single 35S::*CO* line used in Song et al. the expression of *CO* mRNA is relatively weak and much weaker in the phloem companion cells than in our *SUC2*::*CO* line. Thus a reduction in CO protein level by the *fkf1* mutation might be more evident in the low expressing *35S:CO* line than in our *SUC2*::*CO* line. We are therefore careful not to argue that *fkf1* does not reduce CO levels, just that this reduction could not be detected in our material. However, as we could detect a reduction of CO protein in the *prr* quadruple mutant, we are confident that the PRRs have a stronger role in CO stabilization than FKF1. On p.8, where we describe these results, we conclude only that PRRs have a stronger effect than FKF1 in stabilizing CO when it is strongly expressed from SUC2 promoter in the phloem companion cells, where CO is also expressed in WT plants.

6) PRR-CO interaction on figure 5. The control used in panels C and D should not be an empty vector but GFP. The data presented here does not allow discriminating between an interaction with PRRs or GFP. It is very difficult to see anything for the PRR:CFP images in panel A. Could the authors present images that are more clear?
Response: In this new version we include data of co-IP with another protein as the negative control. In this experiment, as discussed above, we used TRB3 protein fused to YFP, showing that HA:CO is co-immunoprecipitated with TOC1:GFP but not with TRB3:YFP (Fig. S7 and mentioned on page 12 in the Results).

7) For all the figures from the paper it should clearly be stated what exactly is being shown for the quantified gene expression values and protein level values. This is not always clear from all the figure legends. Do the authors show averages from biological repeats or averages from technical repeats of a representative experiment? N=? errors shown as SD or SE?

Response: The reviewer is correct that this was not always clear in the original version. In the revised version we have included this information specifically in figure legends and in the methods explain how the data from biological replicates of RT-PCR and Westerns were combined to compile the graphs shown.

8) Also regarding gene expression data. Gene expression is typically shown as relative data (not absolute number of transcript which is very hard to get). For the panels (most of them) when data for multiple genotypes are shown what exactly do the authors show? Expression relative to one time point in one genetic background? Please make this clear in materials and methods and/or in the legend of the first figure showing such data.
Response: The reviewer is correct that this was not clear previously. These relative values are calculated against those of internal controls, *PP2a* mRNA for mRNA analyses and Histone 3a for protein analyses. We include this information now in the figure legends. We also now add information on the method of data analysis that we used for combining biological data sets for the expression studies in the method section on page 18. Briefly, after normalizing all the values of expression of tested genes in all the genotypes with the values of *PP2a*, the sample with highest expression in each replicate was set to 1, and the values of all other samples were re-calibrated based on this assignment. After that we averaged the renormalized values within the biological data sets. Thus the value of "1" on the Y-axis in the graph of each expression data represents the highest expression level of the tested gene among all the samples.

Minor comments

1) A map of the CO fragments used in panel 5D would be useful
Response: In the revised version we include a panel that indicates fragments of CO protein used for the co-IP.

2) Review bibliography for some inconsistencies
Response: We checked all the bibliography.

3) The text often refers to significant differences between genotypes but this is not tested statistically, please clarify.
Response: Yes, in the original version some of the wording might have confused biological significance and statistical significance. We have tried to clarify that throughout in the revised version.

Referee #3:

The manuscript by Hayama et al., focuses on the role of PRRs controlling CONSTANS protein stability under long day conditions. The authors use a series of mutants to examine gene and protein accumulation under long days and particular light conditions. The authors also use FRET and protein co-immunoprecipitation in transient assays to demonstrate the interaction between the PRRs and CO. Furthermore, chromatin immunoprecipitation assays reveal that PRRs bind to the FT promoter. As stated, the main conclusion of the manuscript is that: "the diversity in the timing of expression of the PRRs allows CO to accumulate at specific times during the day to generate the typical LD-specific accumulation pattern of CO".

The general topic of the manuscript is highly relevant and the main claim, if fully demonstrated, is significant and sound. However, due to the complexity of the PRR and flowering regulatory networks, some of the conclusions are arguable and require additional experiments while other parts of the data do not fully support the author´s claims and present some inconsistencies that need to be further clarified.

Response: We appreciate the reviewer's interest in the manuscript and suggestions for improvements.

Specific points:

1.
In Figure 1, the authors show the uncoupling of CO and FT mRNA accumulation in plants mis-expressing PRRs. The uncoupling effect is clear on Figure 1C and D but not so much in A and B, as the reduced expression of CO in prr79 or prr57 before dusk correlates quite well with the reduced expression of FT.
We agree that uncoupling between *CO* and *FT* mRNA accumulation is clearer in *PRR* overexpressors (Figure 1) than in *prr7prr9* and *prr5prr7* (Figure 2). However, *FT* mRNA is more strongly reduced in these *prr* mutants than *CO* mRNA (especially at ZT16), as in *prr7prr9 FT* mRNA level is approximately one-tenth of that in WT whereas the *CO* level is identical to the WT level. In *prr5prr7* the *FT* level is almost undetectable whereas the *CO* level is one half of that in WT. However, these marked reductions in *CO* mRNA in *prr* mutants explain why in subsequent experiments we used the heterologous *SUC2* promoter to express *CO* mRNA and then follow the effects of *prr* mutations on CO protein stability.

2.
It is also worth noting the up-regulation of CO at dawn in prr79 as compared to the down-regulation of prr57 at this time point. Therefore, the results indicate that the PRRs have a very important role regulating the transcription of CO. This notion should be taken into consideration when drawing conclusions about the role of PRRs regulating photoperiod through CO function. Also, based on the sequential regulation among the PRRs, it would be important to check the expression of all the PRRs in the mutants and over-expressing lines (e.g. the expression of other PRRs will be affected in PRR5-ox lines).
Response: The reviewer is correct that the PRRs influence *CO* transcription, and this was previously published (Nakamichi et al., 2007). However, our paper seeks to establish that PRRs have an additional role in CO protein stability. We take the reviewers point that we should not give the impression that the PRRs do not influence *CO* transcription and only act through affecting CO protein stability, so in response to this comment we specifically mention the effects of PRRs on *CO* transcription, for example in the final model Figure 7. In addition, in the new version we deleted a sentence "However, the effects of PRRs on *CO* transcription are likely to be indirect by, for example, controlling transcription of upstream components such as the *CDFs* (Nakamichi et al., 2007)." from the discussion section, as this sentence perhaps potentially weakens the message about the significance of the PRR mediated mechanisms that control *CO* transcription. Also in response to the reviewer's comment we added data that show the expression patterns of *PRR9*, *PRR7* and *TOC1* mRNAs in the *PRR5*-ox line, together with *PRR9* and *PRR7* mRNAs in *toc1prr5* plus *PRR9* mRNA expression in *toc1prr5prr7* (Figure S10). The latter results show that the diurnal patterns of *PRR7* and *PRR9* mRNA are not strongly affected in *SUC2*::*HA:CO toc1prr5*, supporting the idea that diurnal activities of these genes are maintained in this line. We mention this information in the Discussion on page 14-15. Also, the peak time of *PRR9* mRNA expression is maintained in *SUC2*::*HA:CO toc1prr5prr7* although the level was higher than in *SUC2*::*HA:CO*. So the lower HA:CO level in *SUC2*::*HA:CO toc1prr5prr7prr9* than in *SUC2*::*HA:CO toc1prr5prr7* especially observed in Fig. 3A, B and Fig. 4A, B is due to the loss of PRR9 activity at its normal time of expression.

3.
In Figure 2, the authors use SUC2::CO lines to express CO in phloem companion cells and to avoid the interference of an impaired clock. However, these lines might not be quite appropriate for these purposes as the expression of SUC2 rhythmically oscillates and clock function in veins was reported to be very important.
Response: As the reviewer indicates, transcript levels of *SUC2* show a circadian rhythm (Michael J. Haydon et al., 2011). However, under the diurnal conditions we used to follow HA:CO protein or flowering time we did not detect a strong effect of impaired circadian clock function in the *prr* mutants on *CO* mRNA expression driven by the *SUC2* promoter. Before using *SUC2*::*CO* we tested expression of *CO* mRNA in this line in constant light following LDs, and found that its levels did not oscillate. Since the *CO* mRNA level is clearly induced during the night and reduced immediately after exposure to light, we suppose that in our light-dark cycles transcript levels of *CO* driven by the *SUC2* promoter mainly respond directly to light and dark, only being weakly affected by impaired clock functions in *prr* mutants. In this revised manuscript we add data that show the expression

pattern of *CO* mRNA driven by the *SUC2* promoter in LL, as well as the expression pattern of *SUC2* mRNA under LD (Fig. S9). This point is mentioned in the Discussion on p.14.

4.
Consistent with these concerns, panel 2A shows a somehow oscillating pattern of CO mRNA but this pattern is not coinciding with the one described for SUC2. Any idea why? Also, are the SUC2::CO lines in a WT background or in co mutant background?
Response: We checked expression of the *SUC2* gene under LDs, which is shown in Figure S9A in this current manuscript. The expression pattern of *SUC2* mRNA is similar to *CO* or *HA:CO* mRNA driven by the *SUC2* promoter in our transgenic lines with a peak around dawn and a reduction in the day. Both *SUC2*::*CO* and *SUC2*::*HA:CO* lines used in this study are in WT background.

5.
It is also intriguing that FT expression in toc1prr5 is almost half of that observed in WT plants (Figure 2F) but this reduced expression does not lead to a flowering phenotype (Figure 2G).
Response: We suppose that the *toc1prr5 SUC2*::*CO* may still contain saturated level of *CO* mRNA and CO protein activity due to the *CO*-overexpression background. Reduced levels of *FT* through these *prr* mutations may not still be sufficient to cause late flowering.

6.
PRR3 is arguably expressed in veins. Did the author check the effect of PRR3 mis-expression on CO stability in phloem companion cells?
Response: We have not tested effects of *prr3* on CO protein abundance, because the effect of *PRR3* on flowering time is still unclear. The *prr3* mutant does not generally show a flowering-time phenotype, and in contrast to overexpression of other *PRRs*, that of *PRR3* was reported to slightly delay flowering (Murakami et al., 2004).

7.
Flowering phenotypes are presented as changes in the number of leaves. Do the authors reach the same conclusions by checking the alteration in the number of days to flowering?
Response: In this new version we add the data showing the number of days to flowering in *SUC2*::*CO prr* mutants. We confirmed that timing of flowering of these lines is consistent with the leaf number (Fig. S2E).

8.
In Figure 3, the authors describe changes in CO protein accumulation on several prr mutants. The authors conclude that the sequential wave of PRR expression regulates CO stability at different phases during the light period of long-day cycles. This is an interesting idea and the conclusion seems to be supported by Figure 3J. However, the pattern of CO accumulation (Figure 3F) shows that there is almost no CO protein around 4-8 hours, i.e. at the time of PRR9, 7 and 5 function. It is then difficult to reconcile a major function for PRRs stabilizing CO protein at a time when there is almost no CO protein. This could be due to differences between the whole plant and phloem companion cells but as the raising phase of CO seems to be advanced in SUC2::HA:CO, the waveforms could be due to clock impairment in the veins of the prr mutants (please see below the comments to Figure S6).
Response: When expressed from the WT promoter in *pCO*::*HA:CO* plants, the reduced accumulation of HA:CO at ZT4 and 8 reflects reduced levels of *CO* transcripts at these times. So we speculate that even though PRR9, PRR7 and PRR5 have the potential to increase the amount of CO protein at these times, this does not occur in wild-type plants because of the lack of *CO* transcripts. Nevertheless, in *pCO*::*HA:CO* plants *CO* mRNA and HA:CO protein do peak in abundance in the morning and evening, and based on their timing of accumulation PRRs could contribute to these peaks. PRR9 could contribute to the morning accumulation whereas PRR7, PRR5 and TOC1 could contribute to the evening accumulation. Therefore, we suppose that the advanced phase of HA:CO accumulation in *SUC2*::*HA:CO* may not be due to the clock being impaired by overexpressing HA:CO in the phloem companion cells, but rather due to very low expression of *HA:CO* mRNA in *pCO*::*HA:CO* at ZT4-8. Circadian rhythms were previously reported not to be affected in 35S::*CO* so overexpression of CO probably does not affect circadian rhythms in the companion cells.

9.
Also, if PRRs sequentially control the timing of CO stability as shown in Figure 3J, then single

mutants should present some phenotypes, which seems not to be the case (e.g. SUC2::HA:CO prr9 shows reduced CO accumulation in the morning but this is not reflected in changes on FT expression (Figure 2D) or in flowering phenotype (Figure 2G).
Response: We speculate that in the *SUC2*::*HA:CO* line HA:CO protein and its activity in terms of inducing flowering time are saturated. and that due to redundancy among PRRs reducing HA:CO level by *prr9* mutation alone is not sufficient to alter flowering time. This idea is consistent with our observation that introducing the *prr9* mutation into *SUC2*::*HA:CO toc1prr5prr7* certainly delays flowering.

10.
The authors also show that the fkf1 mutation did not strongly affect CO accumulation. This is in contrast to previous observations but also it is not consistent with the fact that the ZTL protein family controls the stability of PRRs. Are the authors implying that PRR protein degradation is not regulated by the ZTL family in phloem companion cells?
Response: We suppose that the *fkf1* mutation alone is not sufficient to alter the amount of PRR proteins due to redundancy among ZTL family proteins. *fkf1* mutants were reported to show weaker effects on circadian rhythms than *ztl* (Baudry et al., 2010).

11.
In Figure 4, the authors focus on the light-dependent function of PRRs on CO protein stabilization. The authors perform analyses under BL and FRL. So, under Red Light (RL), regulation is just transcriptional? Are the PRRs not functioning? This is intriguing as the function of some PRRs (e.g. on hypocotyl elongation) is particularly evident under RL. It would be interesting to know whether CO protein is stabilized in PRR-ox lines under RL.
Response: It would be interesting to check the effect of *PRR* overexpression on CO accumulation in red light. However, in this study, we tested the effect of *prr* mutations on the stabilization of CO in blue and far-red light. Red light has the opposite effect and reduces the amount of CO protein (Valverde et al, 2004). The effect of *prr* mutations on hypocotyl elongation in red light might be caused by changes in transcript accumulation of *PIFs* and rather independent from the protein stabilization of CO in flowering.

12.
In Figure 4F, there is detectable protein and a clear oscillation of CO protein in toc1prr579 (red dotted line). However, in Figure 3J, CO protein is quite low throughout the whole cycle (red line). What are the reasons for such discrepancy?
Response: We suppose that this is because in the graph of Figure 3I-L lacks samples at ZT20 where HA:CO abundance is expected to be lower than at other times.

13.
The authors conclude that PRR proteins contribute to light-mediated accumulation of CO. In Figure 4F, it seems that in cop1 toc1prr579 there is less CO protein also during the night (ZT20). Is this reproducible? Are the differences significant among the biological replicates?
Response: Yes. A biological replicate can be found in the supplementary figure section in the previous version. In this new version we combined the replicates and placed it in the main figure section (Fig. 4F). We speculate that there are still unknown factors that control CO abundance in light-dark cycles.

14.
The authors also compare cop1 with cop1toc1prr579 mutant lines to conclude that PRRs stabilize CO by suppressing the capacity of COP1 to degrade CO. The results indicate that the prr phenotype requires the presence of a functional COP1. However, full demonstration that PRRs suppress COP1 function would require additional functional evidence. For instance, did the authors check whether expression of COP1 is affected in the prr mutants? Also, the authors could check CO stability in COP1-ox lines (or inducible ox) and then examine whether over-expression of PRRs enhances the stability of CO. As the main topic of the manuscript relates to PRRs and stability of CO protein, including these experiments might be a bit out of the scope of the manuscript, but if the authors maintain their statement in results and discussion, then compelling evidence showing that PRRs suppress COP1 activity is necessary.
Response: We agree with this comment, and change our original statement in the result section "Together, these results indicate that PRRs stabilize CO by suppressing the capacity of COP1 to

degrade CO." to "These results demonstrate that reduced CO abundance in *prr* mutants requires COP1 activity, and suggests that PRRs stabilize CO by suppressing *COP1*-mediated degradation of CO in the light." on page 9. We also change related statements in the discussion section (p15-16).

Regarding the question of whether *COP1* mRNA and COP1 protein alter in *prr* mutants, we propose that these levels are likely not affected because the level of a COP1 target protein HY5 did not change in the *toc1prr5prr7prr9* mutant. We still do not know how the genetic epistatis between *prr* and *cop1* is generated, so will further analyze the relationship between PRRs and COP1 in controlling CO accumulation. We have not checked whether overexpression of a PRR can enhance accumulation of CO in a COP1 overexpressor.

15.

The authors also checked hypocotyl elongation and whether other COP1 targets (such as HY5) might be also affected in the prr mutants. Quite surprisingly, they found that the long hypocotyl phenotype in toc1prr5prr7prr9 was observed only under LDs and SDs but not under continuous light. This is difficult to reconcile with previous reports showing for instance a clear hyposensitive phenotype in several prr mutants under RL [e.g. Nakamichi et al., Plant Cell Physiol. 46(5): 686-698 (2005)].

Response: One possibility is that in this and other related papers relatively low intensities of red light were used for measuring hypocotyl length, whereas we used high intensity WL. This WL exposure might have overcome the effect of higher accumulation of *PIF4* mRNA on hypocotyl elongation in the *prr* quadruple mutant, fully suppressing its protein function. *PIF4* transcripts are known to be increased in the *prr5prr7prr9* mutant (Nakamichi et al., 2009 and Fig. S5 in the current study).

16.

The pattern of PIF4 expression in toc1prr5prr7prr9 (Figure S5E) is practically the same to that shown for CO in Figure 2A. Is this a mistake?

Response: We checked the low data again and confirm that this panel shows *PIF4* mRNA accumulation.

17.

Figure 5 shows data about the physical interaction between PRRs and CO. These results are interesting but there are a number of questions and issues that need to be addressed:

- At what time was FRET performed? Are there changes in the interaction depending on the time-of-day?

Response: We performed FRET with Arabidopsis protoplasts and *benthamiana* leaves twice for each (Fig. 5 and Fig. S7), confirming the same trends among the results. In the FRET experiment using Arabidopsis protoplasts we generally started checking PRR9 first during the morning and finished all combinations during the evening. We have checked the first PRR9:CFP/CO:YFP sample again at the end of the first experiment within two replicates but did not find obvious changes in the FRET signal.

- If light stabilizes CO protein trough PRR function: Do you see interaction in the dark?

Response: This is interesting to examine, but in this study we have not checked whether the interaction occurs during the night in Arabidopsis protoplasts or *N. benthamiana* leaves. We focused on showing that the interaction does occur in light when CO protein is stabilized.

- Some PRR proteins (e.g. TOC1) are localized in well-defined nuclear speckles. These speckles are not clearly visible in Figure 5A.

- Why CO-YFP localization is so different in the four panels of Figure 5A?

Response: We noticed that among all protoplasts tested there are some cells where speckle formation of PRRs and CO are not very clear and others where they can be clearly detected. Since in *N. benthamiana* almost all of the transgenic cells exhibit clear speckle formation of PRRs and CO, lower frequency of speckle formation that we observed might be specific to protoplasts.

- If PRRs stabilize CO protein, then its accumulation should be overall higher in double PRR/CO compared to single CO transformation. Do you see that?

Response: We have not analyzed CO accumulation in PRR overexpressors. However, in this new version we add a ChIP data showing that overexpression of *TOC1* in *SUC2*::*HA:CO* enhances

enrichment of the *FT* promoter with HA:CO on the ChIP experiment (Fig. S8), implying its increased accumulation by *TOC1* overexpression. Other PRR overexpressor lines with *SUC2*::*HA:CO* have not fully been prepared. We did not specifically test the abundance of proteins in transient assays.

- The Y axis in Figure 5B is labeled as "FRET Efficiency (%)". Does this efficiency reflect the percentage of donor decreased fluorescence before and after acceptor bleaching?
Response: Yes. The efficiency was calculated using the general formula (pre-bleaching – post-bleaching)/ pre-bleaching, and we add this information to the method section in the new manuscript.

- Figure 5C shows that the interaction of CO with PRR9 is clearly lower than with the other PRRs (at least based on the amount of immunoprecipitated proteins). Was this result consistently observed in the different biological replicates? The FRET analyses suggest otherwise.
Response: We cannot definitively explain why these two methods appear to give different relative efficiencies of interaction. However, FRET efficiency is generally affected by other issues than whether the two proteins interact, such as the distance between the fluorophores, which might differ between PRR-CO pairs.

- It is a pity that the authors cannot examine the interaction using stable transgenic Arabidopsis lines because the results using these plants could provide conclusive information about the timing and the possible light-dependent interaction.
Response: We agree with this comment. We first planned to check time-dependent immunoprecipitation of PRRs with HA:CO, creating *SUC2*::*HA:CO/pPRR*::*PRR:GFP*. However, we had difficulties in immunoprecipitating HA:CO from *SUC2*::*HA:CO* lines perhaps because of its low abundance in plant extracts due to its specific expression in phloem companion cells.

18.
In Figure 6 the authors show that PRRs bind to the FT promoter. However, this figure is not properly explained, which complicates the interpretation of the results. For instance, are the numbers 1-4 on the X axis corresponding to sampling at ZT1, 8, 12 and 16? It is not clear in the figure legend. Are the authors expecting a differential binding depending on the time of PRR function? It doesn´t seem the case. The manuscript´s conclusions would be also clearly reinforced if the authors compare the binding of CO to the FT promoter in HA:CO versus HA:CO/PRRs. If PRRs stabilize CO protein, binding could be also enhanced.
Response: We have performed the HA:CO ChIP experiment requested by the reviewer and included the data. As suggested by the reviewer we do detect much stronger binding of HA:CO to *FT* promoter in WT plants than in *prr* mutants, strengthening the conclusion of the manuscript. However, as stated above we deleted the data on PRR ChIP, because the previous ChIP data showing binding of PRRs to the *FT* promoter could not be reproduced.

19.
In Figure S6, the authors attempt to demonstrate that the phenotypes are independent from the clock. However, conceptually, this idea is a bit incoherent with the main conclusion that the timing of PRR function regulates CO stability at different phases during the light period of long-day cycles. Indeed, timing and phase of PRRs are intimately ligated to the clock function. In any case, methodologically, just checking FKF1 or TOE1 expression is not compelling enough to conclude that the phenotypes are independent from the clock. Also, they refer to the prr9 mutant, but this mutant do not show changes on FT expression (Figure 2D) or in flowering (Figure 2G). If the authors want to examine independence from the clock, they could "eliminate" timing for instance by growing the seeds for several days under continuous light (without any previous synchronization).
--- According to these comments we changed our previous statements in the result section "…suggesting that the observed effect of the quadruple *toc1 prr5 prr7 prr9* mutant on HA:CO protein level might be an indirect effect of an impaired circadian clock." for "suggesting that the observed effect of the quadruple *toc1 prr5 prr7 prr9* mutant on HA:CO protein level could be due to altered expression of other clock-regulated genes that affect CO accumulation." (p.10).

We also changed another statement at the same section "…is not an indirect effect of impairment of circadian clock function or changes in *FKF1* or *TOE1* activities but rather reflects a more direct

effect of PRRs on CO." for "…is not an indirect effect of altered expression of clock-output genes such as *FKF1* or *TOE1*, but rather reflects a more direct effect of PRRs on CO.".

We also included additional data showing that in *prr9 SUC2*::*HA:CO FT* mRNA accumulation at ZT1 is lower than *SUC2*::*HA:CO* (Fig. S4). Regarding the flowering time data, we suppose that due to redundancy among PRRs in the *SUC2*::*HA:CO* background where activity of HA:CO is saturated, the single *prr9* mutation may not be enough to cause a change in flowering time in this *CO* overexpressor. We have not tested HA:CO abundance in our lines under LL, since we suppose that even if the clock does not oscillate, mutations in the *prr* genes may still affect expression of the downstream genes even in this condition.

20.
The Discussion is interesting but as mentioned in the comments of Figure 4, the authors should either provide compelling evidence that PRRs stabilize CO by suppressing the capacity of COP1 to degrade CO or decrease the tone of their conclusions accordingly with the actual results that they provide. Also, the authors should include in Discussion (and/or Introduction) the recent findings by the Imaizumi´s lab: Distinct roles of FKF1, Gigantea, and Zeitlupe proteins in the regulation of Constans stability in Arabidopsis photoperiodic flowering. Song YH, Estrada DA, Johnson RS, Kim SK, Lee SY, MacCoss MJ, Imaizumi T. (Proc Natl Acad Sci U S A. 2014 Dec 9;111(49):17672-7).
Response: As shown in our comment to point 14, we changed the related statements on CO stabilization by suppressing *COP1* function by *PRRs*. We also add in the Introduction section on page 4 a statement about GI and ZTL functions to control CO abundance in Arabidopsis and the reference is mentioned.

Other comments:

1. The results should be accompanied by statistical significance of biological replicates. The number of biological replicates and the number of samples examined in each replicate should be clearly stated.
Response: The reviewer is correct, and this was raised by the other reviewers. As mentioned above, we now provide all of this information in the new figure legends and the method section.

2. Many panels in Supplemental Figures are not described or even mentioned in the text (e.g. Figure S2B, S5C, S6D, E, F, etc).
Response: Several panels have been moved among the figures in the new version. But we have made an effort to ensure that all panels are cited in the text of the new version.

3. Please revise grammar and typos throughout the manuscript.
Response: In this new version we checked grammar and typos.

4. Figure 1 mostly refers to the uncoupling between CO mRNA and FT mRNA. Based on previous reports about the importance of CO protein stability, the novelty of this figure is relatively minor in the context of the whole manuscript. Therefore, if needed, this figure could go to supplemental.
Response: According to the reviewer's comment, in the new version we move the original Figure 1A/B showing expression of *CO* and *FT* in the double *prr* mutants to the supplementary section (Fig. S2).

5. In the Introduction (page 4) when it says: "This coincidence between CO protein and light exposure allows stabilization of the protein, causing CO to accumulate under LDs and achieving recognition of LDs (Fig. S1) (Valverde et al., 2004)" it should say: "This coincidence between CO mRNA..., right?
Response: In this revised version we change "CO protein" for "*CO* mRNA" according to the comment.

6. The authors state several times in the manuscript that the components and mechanisms from light to flowering "remain unclear". This statement is a bit misleading. The myriad of components and mechanisms thus far described are clear, maybe it would be more accurate to say that they are incomplete (or similar).

Response: In this new version we change statements related to this comment especially in the introduction and discussion part, stating "…. is not fully understood".

7. In Materials and Methods, the light and temperature conditions in which the plants were grown in the different experiments should be specified.
Results: In this version we add light and temperature conditions in the method section on p.17-18 according to the comment.

We would like to take this opportunity to thank all of the reviewers and the Editor for their detailed reading of the manuscript and suggestions for improvement. We do believe that the resulting revisions have greatly improved the manuscripts.

2nd Editorial Decision 04 January 2017

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are all broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address the remaining concerns of the referees and to provide a final version of your manuscript. Please note that the manuscript length is not an issue (referee #3).

REFEREE REPORTS

Referee #1:

In this manuscript authors show that the circadian clock PRR proteins directly interact with CONSTANS and contribute to stabilization of this factor during the day, by competing for COP1-mediated degradation. Authors show that FT expression levels are reduced in higher order prr mutants, which correlates with delayed flowering time, and these effects are largely independent on CO transcription levels. A direct effect on CO protein stabilization is confirmed by using SUC2::HA-CO lines, where the toc1 prr5 prr7 prr9 mutations strongly reduce HA-CO protein levels. Authors show that PRRs contribute to light-mediated accumulation of the CO protein, with reduced blue and far-red light mediated stabilization of the CO protein observed in the toc1 prr5 prr7 prr9 background. Destabilizing effects of the prr mutations are suppressed in the cop1 background, indicating that PRRs stabilize CO by suppressing COP1-mediated degradation of this protein in the light. They show that this stabilizing effect is not observed for HY5, excluding a general effect on COP1 activity. By using FRET and co-IP studies it is shown that PRRs directly interact with CO and it is provided additional evidence showing that stabilization of the CO protein correlates with increased binding to two conserved CORE elements in the FT promoter and the second intron region.
Overall the MS is notably improved in this revised form, where authors addressed most of the raised concerns. If any it is surprising the finding that PRRs and CO interact via the CCT domain, which is the domain involved in DNA interaction. As PRRs share also a conserved CCT domain it would be logical to assume that the CCT domain of the PRR proteins aids at DNA recognition. However, authors could not reproduce previous results showing that PRRs bind the FT promoter. Some additional discussion on the proposed model for the observed regulation might still be required.

Referee #2:

I am satisfied with this revised version of the manuscript. I have a few minor comments that can all be dealt with in writing. The only exception is comment 1 that requires additional statistical treatments and explanations throughout this paper.

Comments

1) Generally speaking statistical treatment of the data should be improved. (i) on no figure is there any mention of what sample is statistically different from controls, this is a bit difficult for figures with time course but should be clearly indicated on 2E, 4I, 5B, 6B, S2E, S3F, S4A S6F and S8. For all those define the statistical test used and the p value indicating significant difference from control. (ii) Statistics should be homogenized, on some figures the authors show mean +/- standard error, on others mean +/- standard deviation (e.g. compare legend of Figures 3 and 6). If there is a good reason for such different treatments please indicate. (iii) for RT-qPCR data the authors indicate that the data are means of 2 biological replicas. However, I suspect that technical replicas for the RT-qPCR were performed, if that is true please clearly indicate.

2) The general concept that PRRs affect flowering time through circadian regulation of CO transcription should be made clear earlier in the paper. I would actually suggest doing this in the abstract. What is clearly shown here is that in addition to this role on CO transcription, PRRs also regulate CO protein stability. This would be particularly useful for the non experts.

3) Can the authors please comment on Figure 3B/C. At ZT0 and ZT20 there is a big difference in FT expression comparing SUC2::HA:CO and SUC2::HA:CO toc1prr5prr7prr9 (panel C), however on panel B there is no obvious difference in CO accumulation (at ZT0 and ZT20). Any suggestion of what might be happening?

4) Based on the author's data would it be fair to say that morning CO expression does not appear to play an obvious role in the regulation of flowering time in their growth conditions? In the prr9 mutant morning CO expression is pretty much gone (3I) but flowering is normal (2E). Please comment.

5) Several papers were recently published indicating that PRRs can be associated with chromatin (e.g. Zhu et al., 2016 Nat Comm; Soy et al., 2016 PNAS; genome-wide studies on PRR chromatin binding). Although the authors do not provide evidence that PRRs bind to the FT promoter (might be due to the very small number of cells where this happens), the authors should briefly mention this possibility. Just a couple of phrases should suffice.

6) On page 10 the authors write that PIF4 protein accumulation requires shade or darkness and cite (Leivar 2008 and Nozue 2007), neither of those papers shows shade regulation of PIF4, this was shown in Lorrain et al., Plant J. 2008.

7) Page 13 regarding HA:CO ChiP in the 35S::TOC1 line. The authors do not show that HA:CO levels are higher in the 35S:TOC1 line. Please rephrase or show the data.

8) The difference between HY5 accumulation and HA:CO accumulation in the toc1prr mutant background might be related to the fact that HY5 is expressed broadly while HA:CO is restricted to the vasculature. As there is evidence for cell type specific clocks the authors may want to mention this point in the discussion.

Referee #3:

In the revised manuscript, the authors have included new data and text that answer many of the concerns raised in the original manuscript. However, there is still an unresolved issue related to the timing of PRR function on CO protein stability (point 8). In their response, the authors argue that "PRR9 could contribute to the morning accumulation whereas PRR7, PRR5 and TOC1 could contribute to the evening accumulation". While PRR9 might function close to dawn - even though prr9 mutants do not have a clear flowering phenotype (Fig. 2E) and the other PRRs are expressed at later time points - the role of PRR7 in the evening is very unlikely based on its pattern of expression (peak at ZT7 and clearly reduced expression in the evening). As stated in the previous revision, it is difficult to reconcile a major function for PRRs stabilizing CO protein at a time when there is almost no CO protein in WT plants. Although the notion of the sequential function of PRRs shaping CO

protein waveform is very appealing, the presented results do not fully demonstrate this idea.

Other points:

1.As stated in the previous revision, if manuscript length is an issue, Figure 1 can go to supplemental.

2.In response to point 5, the authors speculate that "reduced levels of FT through the lower order prr mutations may not still be sufficient to cause late flowering". Maybe this explanation can be included in the text.

3.In response to point 6, the authors state that overexpression of PRR3 slightly delays flowering. However, this argument could be also used for TOC1, as TOC1 over-expressing lines are also late flowering.

2nd Revision - authors' response 31 January 2017

Response to reviewers' and editor's comments on revised version
Below, we respond to each of the points raised by the editor and reviewers on the first revised version of our manuscript. We describe also the changes we have made in the second revised version.

Reviewer 1.
This reviewer comments that "overall the MS is notably improved in this revised form, where authors addressed most of the raised concerns" however mentions that "some additional discussion on the proposed model for the observed regulation might still be required".
Response: As requested by the reviewer and by reviewer 2 (point 5), we have edited the Discussion. Specifically, we have mentioned in the Discussion on page 14 that PRRs might have additional roles in binding to the FT promoter co-operatively with CO to control transcription.

Reviewer 2.
This reviewer comments that "I am satisfied with this revised version of the manuscript" however requests additional statistical treatment of some data and some further editing of the text. We deal with each of the points raised by the reviewer below:

1. The reviewer asks for an improvement of the statistical analysis of data in three areas (i) mention what sample is statistically different from controls in figures 2E, 4I, 5B, 6B, S2E, S3F, S4A S6F and S8. Also for these figures define the statistical test used and the p value indicating the significant difference from the control. (ii) Consistently use standard error or standard deviation rather than both (e.g. in Figures 3 and 6). Alternatively, if there is a good reason for such different treatments please indicate. (iii) For RT-qPCR data the authors indicate that the data are means of 2 biological replicas, but the reviewer asks that we also indicate how many technical replicates were used.

   Response: (i) We have indicated which differences are significant with asterisks and P values in Figure 2E figure and legend; legend to figure 4I; in Figure 5B figure and legend; in Figure 6B figure and legend; in Figure EV1E (previously S2E) and legend; in Figure EV2F (previously S3F) and legend; in Figure EV3A and B (previously S4A and B) and legend; in Figure S2F (previously S6F) and legend; in Figure EV6 (previously S8) and legend. (ii) We have dealt with this issue, changing the use of standard deviation for standard error to use the same statistical values in all cases. (iii) We have added information on the number of technical replicates in the qRT-PCR data in the Figure Legends.

2. The reviewer asks that the general concept that PRRs affect flowering time through circadian regulation of *CO* transcription is made clear earlier in the paper, even in the Abstract, and emphasise earlier that what is shown here is an additional role for PRRs in regulating CO protein stability.

Response: We have followed the reviewer's suggestion and in the rewritten Abstract we have specifically mentioned the originally characterized role of PRRs as clock components that influence CO transcription.

3. The reviewer asks us to comment on Figure 3B/C because at ZT0 and ZT20 there is a big difference in *FT* expression comparing SUC2::HA:CO and SUC2::HA:CO toc1prr5prr7prr9 (panel C), however in panel B there is no obvious difference in CO accumulation (at ZT0 and ZT20).

   Response: We have not investigated this issue, although it is possible that PRRs have an additional function in inducing FT expression by interacting with CO, independently from their roles in stabilizing CO. However, since FT mRNA level at the end of the night is generally very low in SUC2:CO and reduction of FT in toc1 prr5 prr7 prr9 SUC2::HA:CO at this time is not observed in SUC2:CO, we assume that this occurs specifically in this SUC2::HA:CO line. In this line HA:CO activity might be very high, and its saturated level might still cause accumulation of FT mRNA even during the night. However, this does not affect our major conclusion that PRRs stabilize CO or HA:CO.

4. The reviewer asks us to comment on whether morning CO expression plays an obvious role in the regulation of flowering time in our growth conditions, because in the prr9 mutant morning CO expression is pretty much gone (3I) but flowering is normal (2E).
   Response: The reviewer indicates figures in which the effect of prr9 on CO or HA:CO activity is measured in plants carrying the SUC2 promoter fusions. These data show that *prr9 mutation is enough to reduce CO protein level but not to cause a change in flowering time in SUC2::CO (Fig. 2E) and SUC2::HA:CO (Fig. EV2F) backgrounds. We believe that in these backgrounds CO activities are very high at other times of the day and that this causes saturated activity of the downstream flowering-time pathway so that the effect of prr9 on flowering cannot be detected. The prr9 mutant without these transgenes is reported to exhibit a late flowering phenotype (Nakamichi et al., 2005), and we think that this is consistent with our idea that the morning peak of CO protein also contributes to flowering.*

5. The reviewer requests that we briefly mention in a couple of phrases the possibility that the PRRs bind to the FT promoter in the context of recent publications showing that PRRs bind chromatin.

   Response: *We have now specifically mentioned in the Discussion on page 14 the possibility that PRRs also bind to the FT promoter and contribute to FT transcription in cooperation with CO and have included the two references mentioned by the reviewer.*

6. The reviewer requests that on page 10, where we mention that PIF4 protein accumulation requires shade or darkness, we cite Lorrain et al., Plant J. 2008.
   Response: We have followed the reviewer's suggestion and cited this reference on p.10 in the Results.

7. The reviewer points out that on Page 13 regarding HA:CO ChiP in the 35S::TOC1 line, we do not show that HA:CO levels are higher in the 35S:TOC1 line and requests that we rephrase or show the data

   Response: In the revised manuscript we have mentioned specifically at this point in the Results on p.13 that we have not tested the effect of TOC1 overexpression on HA:CO accumulation in SUC2::HA:CO and rephrased the conclusion as requested by the reviewer.

8. The reviewer suggests that we mention in the Discussion that the difference between HY5 accumulation and HA:CO accumulation in the toc1prr mutant background might be related to the fact that HY5 is expressed broadly while HA:CO is restricted to the vasculature, particularly as there is evidence for cell type specific clocks.

   Response: We agree with the reviewer that this is a possibility. We have now specifically mentioned in the Discussion on page 16 that PRRs might also have a role in controlling CO abundance through regulation of a general COP1 activity specifically in the vasculature, which also potentially changes expression of HY5 as well in this tissue, and that we might not have been able to detect alteration in HY5 level specifically in the vasculature because HY5 is also broadly expressed in various tissues in A. thaliana. However, we make the additional argument that PRRs are also broadly expressed so if they did regulate HY5 levels in the vasculature one might expect them to have a similar function on HY5 outside the vasculature (Fujiwara et al., 2008; Para et al., 2007).

Reviewer 3.
This reviewer comments that **"**in the revised manuscript, the authors have included new data and text that answer many of the concerns raised in the original manuscript" however also raises a few issues that should still be dealt with.

1. The major point is that a role of PRR7 in the evening in CO stabilization "is very unlikely based on its pattern of expression (peak at ZT7 and clearly reduced expression in the evening). As stated in the previous revision, it is difficult to reconcile a major function for PRRs stabilizing CO protein at a time when there is almost no CO protein in WT plants.

   Response: Although the reviewer is correct that the diurnal transcript level of PRR7 peaks at around ZT7, its protein level peaks later at approximately ZT12 (Fujiwara et al., 2008; Farré et al., 2007). Therefore, we believe that PRR7 could still contribute to the evening peak in CO in WT plants and have retained this argument.

2. The reviewer mentions that if length is an issue, Figure 1 could be deleted. However, the editor mentions that there is no need to shorten the manuscript.

3. The reviewer mentions that in response to point 5 of our previous rebuttal, we speculate that "reduced levels of FT through the lower order prr mutations may not still be sufficient to cause late flowering". Maybe this explanation can be included in the text.

   Response: As requested by the reviewer, in the new version of the manuscript, we described in the Results section on page 7 that toc1 prr5 SUC2::CO might not flower much later than SUC2::CO despite having less FT mRNA because in toc1 prr5 SUC2::CO the levels of FT are still so high that their effect on flowering is close to saturation. Therefore, the further increase in FT levels in SUC2:CO would not accelerate flowering much more.

4. The reviewer mentions that in response to point 5 of our previous rebuttal, we state that overexpression of PRR3 slightly delays flowering and the reviewer points out that this argument could also be used for TOC1, as TOC1 over-expressing lines are late flowering.

   Response: The reviewer is correct, and analysing the role of PRR3 in flowering-time control in the future would be interesting. In this study, however, we did not analyse PRR3, since we felt that the contribution of PRR3 to flowering-time control of wild-type plants was less clear than for other PRRs. On the other hand, the contribution of TOC1 to flowering-time control of wild-type plants seemed more pronounced than that of PRR3 because, for example, it is known that loss of TOC1 function clearly causes late flowering and reduction in FT transcripts, particularly when the mutation was combined with prr5 (Ito et al., 2008). This clearly leads to speculation that TOC1 has a role in promoting

flowering in wild-type plants redundantly to PRR5, which we further test in our manuscript.

Finally, we would like to thank the reviewers and editor for their further engagement with our work and their suggestions for improvements. We are sure that these have increased the quality of the paper and improved its attractiveness to the reader.

3rd Editorial Decision 01 February 2017

Thank you for submitting your revised manuscript to us. I appreciate the introduced changes and I am happy to accept your manuscript for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: George Coupland
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-93907R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes for the experiments to analyze flowering time, mRNA expression and protein abundance in the current paper were determined based on our knowledge acquired from our previous experience and from those used by other laboratories in similar studies. We confirmed that the sample sizes that we used in this study were adequate by performing biological replicates and obtaining similar results.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In this manuscript data with animal sources are not included.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All populations were exposed to similar growth conditions and control and experimental populations were grown in the same environments. No specific steps were taken to minimize bias but as each experiment involved relatively large populations of genetically identical individuals we do not expect this to be a problem.
For animal studies, include a statement about randomization even if no randomization was used.	In this manuscript data with animal sources are not included.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Many experiments were performed by more than one of the experimentalists reducing the possibility for subjective bias. Also the data were shared among experimentailists.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In this manuscript data with animal sources are not included.
5. For every figure, are statistical tests justified as appropriate?	In this study we have performed statistical methods that we and others have used used in other studies. This point was discussed extensively with referees and now we show standard error for all data where error bars are shown and we indicate which differences are significant with P values in the legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We confirmed that data show normal distribution and are suitable for the tests used.
Is there an estimate of variation within each group of data?	In this study variation has been shown by either standard error.

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Is the variance similar between the groups that are being statistically compared?	We have confirmed that the variance is similar between the sample groups of each experiment.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	HA detection and co-IP: Anti-HA-Peroxidase, High Affinity (Roche; 12013819001), GFP detection and co-IP: Anti-GFP antibody-ChIP Grade (Abcam; ab290), ChIP assay: Anti-HA tag antibody-ChIP Grade (Abcam; ab9110)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have not used cell lines.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have not used animal sources in this study.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have not used animal sources in this study.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We have not used animal sources in this study.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The current study is not related to human research.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We do not provide data for which accession codes are required..
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	In addition to including primary data to the Main Figure section, we have included additional datasets that are also central and integral to our study in the Supplementary Figure section.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	In this study data that access to human clinical and genomic datasets are not included.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	In this study datasets with which primary and referenced data need to be cited do not exist.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	In this study data based on or including computational models do not exist.

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	This study does not fall under dual use research restrictions.