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The cold response regulator CBF1 promotes *Arabidopsis* hypocotyl growth at ambient temperatures

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1st Editorial Decision 11th Nov 2019

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the work and the topic. However, they also raise a number of substantial and partially overlapping concerns, especially regarding the in vivo relevance and context of the described CBF1-PIF crosstalk and potential alternative explanations of the observed phenotypes that would have to be addressed before they can support publication here. From my side, I judge the referee comments to be generally reasonable, and find that resolution of these issues in the revised manuscript is crucial for further consideration here. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

REFEREE REPORTS:

Referee #1:

The manuscript reports on a novel function of the cold responsive C-repeat/DREB binding factor1 (CBF1) as a negative regulator of photomorphogenic development in Arabidopsis. Authors show

that CBF1 directly binds to CRT/DRE and GCC motifs in the PIF4 and PIF5 promoters to activate expression of these etiolation-promoting factors, in addition to bind the PRD domains of phyA and phyB, and compete for interaction of the biologically active phyB Pfr form with PIF4 and PIF5. CBF1 thus promotes PIF4 and PIF5 protein accumulation in the light via both transcriptional and protein stabilization mechanisms, these findings revealing that these factors form a complex regulatory loop implicated at integration of light and low temperature signals.

PIFs, including PIF3, PIF4 and PIF7, were previously reported to repress CBF gene expression under low temperatures. Likewise, CBF over-expression was previously reported to lead to growth retardation, although authors show in this work that cbf1 mutants and CBF1-OE lines respectively display shorter and taller hypocotyls than the wild-type in the light. This phenotype is not observed in darkness, therefore suggesting a function of CBF1 in negative regulation of light-induced responses, and is opposite to CBF2 and CBF3 over-expressers which displayed reduced growth irrespective of light conditions, consistent with previous reports. CBF2 and CBF3 were actually established to play a more critical role in cold acclimation and COR gene activation than CBF1, hence suggesting that this factor may have evolved a distinct function to CBF2/3 in regulating plant growth and development.

The work shows that CBF1 transcript levels are induced in response to light, and that light promotes CBF1 protein stabilization, although light activation of this gene is far weaker that its response to cold (10-20 fold versus >1000- fold). CBF1 transcript levels are also strongly decreased in red light in the phyA, phyB and double phyA phyB mutants, indicating that these photoreceptors are required for light activated CBF1 expression. Moreover, cbf1 loss of function partly suppressed the elongated phenotype of phyA and phyB seedlings in the light, thus supporting a function of CBF1in increased hypocotyl elongation of these mutants. Authors actually show that PIF4 and PIF5 protein levels are drastically decreased in white and red light in cbf1 mutants consistent a role of CBF1 in positively regulating PIF4 and PIF5 protein accumulation in the light. Also, cbf1 pif4 pif5 and pif4 pif5 mutants displayed identical hypocotyl lengths in the light, demonstrating a role of PIF4 and PIF5 downstream of CBF1. Furthermore, 35S:PIF4 cbf1 lines display in W and R light shorter hypocotyls than 35S:PIF4 seedlings, which correlate with lower levels of the PIF4 protein in 35S:PIF4 cbf1 seedlings in the light, but not in the dark. Overall, these findings strongly support a role of CBF1 in regulating PIF4 protein abundance in the light by directly binding to conserved CRT/DRE and GCC motifs in the PIF4 promoter and mediating activation of this gene in addition to play a role in stabilization of the PIF4 protein in the light, by to competing for phyB-PIF4 interaction. These are novel and interesting results that may merit acceptance for publication. However, a major drawback of the manuscript is that all sort of experimental evidence concerning the biological context in which this regulation would be relevant is missing. Authors speculate that this regulation may play an important role during dark-to-light transition on seedlings emergence from soil but experimental evidence supporting this function is not provided. Authors generated multiple mutant and OE lines used to analyze PIF4 gene expression and PIF4 protein abundance and diurnal hypocotyl elongation studies under different light and temperature regimes using these materials shall be key to demonstrate the importance of this regulation. For instance, it is reported that enhanced hypocotyl elongation of PIF4-OX seedlings is suppressed at lower temperatures and it would be key assessing growth of the 35S:PIF4 and 35S:PIF4 cbf1 lines under cold temperatures which strongly activate expression of the CBF1 gene. Showing that CBF1ox lines are partially resistant to growth suppression at 17ºC may also strongly reinforce significance of the work.
Additional points that require to be further addressed are:
1) Light mediated stabilization of CBF1 is stronger in FR than R or W light. Also, CBF1 seems to more selectively interact with phyA than with phyB, in addition to CBF1-phyB physical interaction not to be reversed in response to a FR treatment. These observations suggest that CBF1 mediated PIFs protein stabilization might be even more relevant for phyA-interacting PIFs than for PIF4 and PIF5. As such, relative importance of both phytochromes in the photomorphogenesis suppressing activity of CBF1 requires to be further discussed.
2) Surprisingly many of the differentially expressed genes in cbf1 mutants and CBF1-ox seem to show the same induced or repressed pattern in both genotypes. This would suggest that CBF1 is subjected to strong post-transcriptional regulation and this is a relevant aspect that should be further discussed.
3) In addition to PIF4 and PIF5, COP1 was identified as a putative direct target of CBF1. Considering the importance of COP1 in photo-and thermomorphogenesis control this finding deserves to be further commented in the discussion part

Referee #2:

This study describes new functions of a well-known factor called CBF1 and also provides mechanistic detail and how CBF1 controls photomorphogenesis. The authors show hypersensitive phenotype of cbf1 mutant under all light conditions, CBF1-phyA/B interaction and CBF1 regulation of PIF4/5 expression. Although their main conclusions are supported by various experimental data, demonstration of in vivo data would make this a much better story. Here are a few suggestions to improve this study.

1. The major drawback is the lack of robust in vivo interaction between CBF1 and phyA/B in a light-dependent manner. They have presented this data in Fig. 5D, but the quality is very poor. It is known in the field that fusing any tag at the N-terminus of especially phyB makes it inactive. Thus, testing with a GST-fusion at the N-terminus may not display true result especially for the N-terminus.

2. They should also provide in vivo data for competition between CBF1-phyA/B interaction vs PIF4/5-phyB interaction. They have shown this in yeast 3-hybrid assay and also split LUC assays Fig. 7D,E), but in vivo data would strongly support this claim. It is not clear whether the reduction in PIF4/5 level in cbf1 mutant (Fig. 7A) is due to reduced transcriptional induction of PIF4/5 in cbf1 and/or increased degradation of PIF4/5 in the cbf1 mutant background.

3. The authors should look into cotyledon area for these mutants as opposite regulation of hypocotyl vs cotyledon area is an indication of hypersensitive phenotype. Shorter hypocotyl might be due to delayed germination or hormone defect or general sickness. This should be ruled out.

4. The expression analysis is strange as shown in Fig. 2C. For example, FR is responding at 3 hr quite strongly compared to other time points, R light is inducing expression at 12 hrs strongly, but not at other time points.

5. Regardless of the expression of CBF1, the protein is unstable in darkness and stabilized under light. This is a hallmark of COP1-regulated response. They should test whether CBF1 is regulated by COP1 post-translationally. If this is the case, cop1 should display freezing-related phenotype. Previously, it was shown that COP1 is absent in the nucleus at 4C (PNAS), perhaps this allows CBF1 to accumulate at 4C and respond to cold. This is related to the broader picture as they described in the introduction that warm temperature is associated with light and cold is associated with darkness. If CBF1 is unstable in darkness (maybe in cold as well?), how does it respond to cold temperature? There must be a mechanism to stabilize it under cold and dark conditions. Exclusion of COP1 from nucleus at 4C makes sense to stabilize CBF1 and thereby respond to cold. This hypothesis can be tested easily.

6. The authors should discuss previous data in the context of their findings. For example, CBF1-PIF4/5 is forming a negative feedback loop where PIFs repress CBF1 and CBF1 activates PIFs. Perhaps this might fit into the discussion about light and cold responses. The discussion of the manuscript is too long (almost 4 pages). They can cut back and discuss broader pictures of general interest and the significance of their study.

7. Figure 4 shows genetic interaction with phy mutants. The data show that cbf1 suppresses phyA and phyB mutant phenotype, suggesting that CBF1 doesn't function in phy signaling pathway. This makes sense if it is functioning in another pathway and attenuating phy signaling pathway.

8. Fig. 6C should be done in dark vs light conditions for PIF4/5 expression.

Referee #3:

In this manuscript, the authors investigated the potential roles of CBF1, an important regulator of cold responses, during photomorphogenesis. They observed that CBF1 activates the expression of PIF4 and PIF5 genes by directly binding to the gene promoters. Moreover, they found that CBF1

interacts directly with phyB, resulting in the enhancement of the stability of PIF4 and PIF5 proteins. In conjunction with the photomorphogenic hypocotyl growth phenotypes of CBF1-deficient mutants and double mutants lacking both CBF1 and phyB, they concluded that CBF1 integrates light and temperature signals to modulate photomorphogenic hypocotyl growth. The biochemical data are strong and well-presented. While the topic is interesting in the field, the authors need to address some critical points, such as the interpretation of molecular genetic data and the effects of cold temperatures on photomorphogenesis to solidify the conclusion.

Major concerns:
1. Figure 4: Both the cbf1-1 phyA-211 and cbf1-1 phyB-9 double mutants exhibited intermediate hypocotyl growth phenotypes compared to those of individual single mutants, obscuring the functional linkage between CBF1 and phytochromes during photomorphogenesis. In addition, the hypocotyl phenotypes of the single and double mutants are not strong enough to support the role of CBF1 in phytochrome-mediated photomorphogenesis (see hypocotyl phenotypes of less than 10% differences in Figure 1). The authors need to clearly address the ambiguous genetic relationship between CBF1 and phytochromes.
2. Figure 7A: It has been reported in multiple references that PIF proteins are rapidly degraded in the light, which is critical for the induction of photomorphogenesis. The authors found that the levels of PIF4 proteins in Col-0 seedlings were increased in white light or red light conditions compared to those under dark conditions (Figure 7A). The authors need to address this inconsistency. They also need to show quantification of all western data in the figures.
3. The authors concluded that CBF1 integrates cold temperature and light signals into photomorphogenesis. However, no direct experiments on the relationship between cold and light were conducted in this study. Are the photomorphogenic responses influenced by cold temperature treatments? Does the CBF1-medaited regulation of PIF4 and PIF5 play roles during photomorphogenesis at cold temperatures? The CBF1 expression is increased by cold temperatures, and CBF1 enhances hypocotyl growth in the light. On the other hand, hypocotyl growth is suppressed at low ambient temperatures (maybe at cold temperatures as well), which is contradictory to the data in this study. It needs to be clarified whether CBF1-mediated photomorphogenic regulation plays a role in integrating light and temperature signals.

Minor comments
1. English grammar would be improved by language editing.
2. Potential roles of the CBF1-phyB-PIF3/4 in thermotolerance and thermomorphogenesis need to be discussed in more detail.

1st Revision - authors' response 4th Mar 2020

Responses to the reviewers' comments

Referee #1:

The manuscript reports on a novel function of the cold responsive C-repeat/DREB binding factor1 (CBF1) as a negative regulator of photomorphogenic development in Arabidopsis. Authors show that CBF1 directly binds to CRT/DRE and GCC motifs in the PIF4 and PIF5 promoters to activate expression of these etiolation-promoting factors, in addition to bind the PRD domains of phyA and phyB, and compete for interaction of the biologically active phyB Pfr form with PIF4 and PIF5. CBF1 thus promotes PIF4 and PIF5 protein accumulation in the light via both transcriptional and protein stabilization mechanisms, these findings revealing that these factors form a complex regulatory loop implicated at integration of light and low temperature signals.

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expression under low temperatures. Likewise, CBF over-expression was previously reported to lead to growth retardation, although authors show in this work that cbf1 mutants and CBF1-OE lines respectively display shorter and taller hypocotyls than the wild-type in the light. This phenotype is not observed in darkness, therefore suggesting a function of CBF1 in negative regulation of light-induced responses, and is opposite to CBF2 and CBF3 over-expressers which displayed reduced growth irrespective of light conditions, consistent with previous reports. CBF2 and CBF3 were actually established to play a more critical role in cold acclimation and COR gene activation than CBF1, hence suggesting that this factor may have evolved a distinct function to CBF2/3 in regulating plant growth and development.

The work shows that CBF1 transcript levels are induced in response to light, and that light promotes CBF1 protein stabilization, although light activation of this gene is far weaker that its response to cold (10-20 fold versus >1000- fold). CBF1 transcript levels are also strongly decreased in red light in the phyA, phyB and double phyA phyB mutants, indicating that these photoreceptors are required for light activated CBF1 expression. Moreover, cbf1 loss of function partly suppressed the elongated phenotype of phyA and phyB seedlings in the light, thus supporting a function of CBF1in increased hypocotyl elongation of these mutants. Authors actually show that PIF4 and PIF5 protein levels are drastically decreased in white and red light in cbf1 mutants consistent a role of CBF1 in positively regulating PIF4 and PIF5 protein accumulation in the light. Also, cbf1 pif4 pif5 and pif4 pif5 mutants displayed identical hypocotyl lengths in the light, demonstrating a role of PIF4 and PIF5 downstream of CBF1. Furthermore, 35S:PIF4 cbf1 lines display in W and R light shorter hypocotyls than 35S:PIF4 seedlings, which correlate with lower levels of the PIF4 protein in 35S:PIF4 cbf1 seedlings in the light, but not in the dark.

Overall, these findings strongly support a role of CBF1 in regulating PIF4 protein abundance in the light by directly binding to conserved CRT/DRE and GCC motifs in the PIF4 promoter and mediating activation of this gene in addition to play a role in stabilization of the PIF4 protein in the light, by to competing for phyB-PIF4 interaction. These are novel and interesting results that may merit acceptance for publication.

However, a major drawback of the manuscript is that all sort of experimental evidence concerning the biological context in which this regulation would be relevant is missing. Authors speculate that this regulation may play an important role during dark-to-light transition on seedlings emergence from soil but experimental evidence supporting this function is not provided. Authors generated multiple mutant and OE lines used to analyze PIF4 gene expression and PIF4 protein abundance and diurnal hypocotyl elongation studies under different light and temperature regimes using these materials shall be key to demonstrate the importance of this regulation.

Response:

We thank this reviewer for these great comments and suggestions. In response, we compared the hypocotyl growth of wild type (Col), two *cbf1* mutants and two CBF1-OE lines at 22°C, 17°C, and 4°C. Interestingly, we observed that the hypocotyl growth of all materials was moderately inhibited at 17°C, but severely suppressed at 4°C (Fig 6A and B). However, we found that compared with Col, the

cbf1 mutants displayed increased sensitivity, whereas the CBF1-OE lines exhibited decreased sensitivity to hypocotyl growth inhibition at 17°C (Fig 6C), indicating that CBF1 attenuates hypocotyl growth inhibition caused by the lower temperature. Moreover, our immunoblot data indicated that CBF1 plays an important role in promoting PIF4 and PIF5 protein accumulation at 22°C and 17°C, but not at 4°C, with a more prominent role at 17°C than at 22°C (Fig 6E and F). These new data indicated that CBF1 promoted PIF4/PIF5 protein accumulation and hypocotyl growth at ambient temperatures, but not under cold stress.

Based on these new data, we also discussed the biological significance of CBF1 in promoting PIF4/PIF5 protein accumulation and hypocotyl growth under ambient temperatures as follows:

Notably, our data showed that CBF1 protein was barely detectable in the dark, but *CBF1* transcript and protein levels were induced in the light, and this induction was mediated by phyA and phyB (Fig 2 and EV1). Thus, in the light, phytochromes repress hypocotyl growth by inducing phosphorylation and degradation of PIF4 and PIF5 (Nozue *et al*, 2007; Shen *et al*, 2007; Lorrain *et al*, 2008); at the same time, phytochromes induce CBF1 accumulation in the light, which in turn promotes PIF4 and PIF5 protein accumulation (Fig 7). It seems likely that this dual regulation of PIF4 and PIF5 by phytochromes could prevent plants from over-responding to prolonged light exposure.

In addition, our data indicate that CBF1 promotes hypocotyl growth at 22°C and 17°C, with a more prominent role at 17°C than at 22°C (Fig 6). These observations are reminiscent of the fact that *Arabidopsis thaliana*, both winter and summer annual ecotypes, mostly germinate and establish seedlings under relatively low ambient temperatures (spring for summer annuals and fall for winter annuals) (Koornneef *et al*, 2004). We hypothesize that during the dark-to-light transition upon seedlings' emergence from soil, the role of CBF1 may be to maintain proper hypocotyl growth under low ambient temperatures. This may be essential for seedling establishment and vital for survival of plants in changing natural environments. Consistent with this hypothesis, studies of natural *Arabidopsis* populations revealed that in contrast to the various frame-shift mutations or nonsynonymous substitutions found in *CBF2* and *CBF3*, almost no frameshift or premature stop codon has been found in *CBF1* (Kang *et al*, 2013; Monroe *et al*, 2016), suggesting a distinct but pivotal role of CBF1 in plant survival under natural conditions.

For instance, it is reported that enhanced hypocotyl elongation of PIF4-OX seedlings is suppressed at lower temperatures and it would be key assessing growth of the 35S:PIF4 and 35S:PIF4 cbf1 lines under cold temperatures which strongly activate expression of the CBF1 gene.

Response:

We thank this reviewer for this great suggestion. In response, we compared the hypocotyl growth of *35S:PIF4* and *35S:PIF4 cbf1* seedlings grown under LD conditions at 22°C and 17°C, and observed that the hypocotyls of *35S:PIF4* seedlings were longer than those of *35S:PIF4 cbf1* at both temperatures (Fig R1A and B). However, the amount of hypocotyl growth inhibition at 17°C was similar in both lines (Fig R1C). Consistent with this observation, our immunoblot data indicated that the PIF4 protein level in *35S:PIF4 cbf1* was 2/3 of that in *35S:PIF4* seedlings grown at both 22°C and 17°C (Fig R1D). These observations indicated

that CBF1 promoted the accumulation of 35S-driven PIF4 proteins at similar levels at both 22°C and 17°C through post-translational regulation.

However, the hypocotyl growth of all examined materials in our study, including *35S:PIF4*, was inhibited at 17°C, although PIF4 protein levels were higher in *35S:PIF4* seedlings at 17°C than at 22°C (Fig R1). Therefore, we concluded that the molecular mechanisms underlying hypocotyl growth inhibition at 17°C were complicated, and may involve many components and pathways in addition to CBF1 and PIF4.

Based on these data and discussion, we decided not to include Fig R1 in our revised manuscript to avoid confusion.

Figures for Referees not shown

Showing that CBF1ox lines are partially resistant to growth suppression at 17ºC may also strongly reinforce significance of the work.

Response:

We thank this reviewer for this great suggestion. In response, we compared the hypocotyl growth of wild type (Col), two *cbf1* mutants and two CBF1-OE lines at 22°C, 17°C, and 4°C. Interestingly, we observed that compared with Col, the *cbf1* mutants displayed increased sensitivity, whereas the CBF1-OE lines exhibited decreased sensitivity (i.e., partially resistant), to hypocotyl growth inhibition at 17°C (Fig 6C), indicating that CBF1 attenuates hypocotyl growth inhibition caused by the lower temperature. We agree with this reviewer that these data will strongly reinforce the significance of our work.

Additional points that require to be further addressed are:

1) Light mediated stabilization of CBF1 is stronger in FR than R or W light. Also, CBF1 seems to more selectively interact with phyA than with phyB, in addition to CBF1-phyB physical interaction not to be reversed in response to a FR treatment. These observations suggest that CBF1 mediated PIFs protein stabilization might be even more relevant for phyA-interacting PIFs than for PIF4 and PIF5. As such, relative importance of both phytochromes in the photomorphogenesis suppressing activity of CBF1 requires to be further discussed.

Response:

We thank this reviewer for this great suggestion. In response, we discussed the suggested point as follows:

Interestingly, the levels of 35S-driven CBF1 decreased to similar levels in *phyA*, *phyB* and *phyA phyB* mutants in continuous W and R light (Fig EV1C), suggesting that phyA and phyB play non-redundant and similarly important roles in post-translational regulation of CBF1 in the light. The fact that CBF1 interacted with only the Pfr form of phyA but with both the Pfr and Pr forms of phyB (Fig 3D and E) suggested that CBF1 interacted more selectively with phyA than with phyB *in*

vivo. It will be interesting to investigate whether CBF1 might also regulate the protein stability of PIF1 and PIF3, both of which specifically interacted with the Pfr form of phyA as well (Shimizu-Sato *et al*, 2002; Shen *et al*, 2008).

2) Surprisingly many of the differentially expressed genes in cbf1 mutants and CBF1-ox seem to show the same induced or repressed pattern in both genotypes. This would suggest that CBF1 is subjected to strong post-transcriptional regulation and this is a relevant aspect that should be further discussed.

Response:

We thank this reviewer for this comment. Indeed, we observed that *HY5* expression was decreased in both *cbf1-1* and *CBF1-myc* seedlings (Fig S6 of our previously submitted manuscript). However, this pattern was only prominent for *HY5* but not for other examined genes (Fig EV3C and S8 of our revised manuscript). Moreover, we observed that the two *cbf1* mutants and two CBF1-OE lines displayed opposite hypocotyl growth phenotypes under continuous light (Fig 1) and LD conditions at both 22°C and 17°C (Fig 6), opposite changes in *PIF4/PIF5* expression in continuous white light (Fig EV3C), and opposite changes in PIF4/PIF5 protein accumulation under continuous white and red light (Fig 4A) and LD conditions at both 22°C and 17°C (Fig 6E and F). These data suggest that the CBF1 level may not be lower in CBF1-OE lines than in Col due to post-transcriptional regulation.

Because we could not presently explain the down-regulation of *HY5* expression in both *cbf1* mutants and CBF1-OE lines, therefore we decided to remove the *HY5* expression data from our revised manuscript to avoid confusion. The inconsistency of *HY5* in *cbf1* mutants and CBF1-OE lines will be further investigated in future studies.

3) In addition to PIF4 and PIF5, COP1 was identified as a putative direct target of CBF1. Considering the importance of COP1 in photo-and thermomorphogenesis control this finding deserves to be further commented in the discussion part

Response:

We thank this reviewer for this great suggestion. In response, we discussed the suggested point as follows:

In addition, *COP1* expression was shown to be moderately modulated by CBF1 (Appendix Fig S8). Considering the important roles of COP1 in photomorphogenesis (Lau and Deng, 2012), thermomorphogenesis (Delker *et al*, 2014; Hayes *et al*, 2017; Park *et al*, 2017), and cold stress responses (Catalá *et al*, 2011), the relationship between COP1 and CBF1 under ambient or low temperatures needs to be further characterized.

Referee #2:

This study describes new functions of a well-known factor called CBF1 and also provides mechanistic detail and how CBF1 controls photomorphogenesis. The authors show hypersensitive phenotype of cbf1 mutant under all light conditions, CBF1-phyA/B interaction and CBF1 regulation of PIF4/5 expression. Although their main conclusions are supported by various experimental data, demonstration of in vivo data would make this a much better story. Here are a few suggestions to improve this study.

1. The major drawback is the lack of robust in vivo interaction between CBF1 and phyA/B in a light-dependent manner. They have presented this data in Fig. 5D, but the quality is very poor.

Response:

We thank this reviewer for pointing this out. In response, we performed additional co-IP assays and obtained an improved image for *in vivo* interaction between CBF1 and phyA, which is shown in Fig 3D of our revised manuscript. Our co-IP data clearly showed that CBF1 interacted preferentially with the Pfr form of phyA, but with both Pr and Pfr forms of phyB *in vivo* (Fig 3D and E).

It is known in the field that fusing any tag at the N-terminus of especially phyB makes it inactive. Thus, testing with a GST-fusion at the N-terminus may not display true result especially for the N-terminus.

Response:

We thank this reviewer for pointing this out. In response, we performed additional *in vitro* pull-down assays using His-tagged PHYA/B-N (photosensory domain), PHYA/B-C1 (PAS-related domain), and PHYA/B-C2 (histidine kinase-related domain) and GST-tagged CBF1. Our data, shown in Fig 3A and B of our revised manuscript, indicated that GST-tagged CBF1, but not GST alone, was able to pull down His-tagged PAS-related domains of both PHYA and PHYB *in vitro*. These data are consistent with our previous *in vitro* pull-down assays using GST-tagged PHYA/B proteins and His-tagged CBF1.

We strongly agree with this reviewer that fusing any tag at the N-terminus of a full-length phytochrome may affect its Pr/Pfr conformation and function. However, this may not necessarily mean that addition of a tag to the N-terminus of a partial phytochrome fragment will definitely affect its activity, because it was shown that MBP-PHYB-N (MBP tag fused to the N-terminal domain of PHYB) interacted with COP1 and was polyubiquitinated by COP1 *in vitro* preferentially in its Pfr form (Jang *et al*, 2010). Therefore, we decided to show our previous *in vitro* pull-down assay data in Appendix Fig S7 to further prove that CBF1 could interact with the PAS-related domains of both PHYA and PHYB *in vitro*, and this interaction was not affected by different fusion tags.

2. They should also provide in vivo data for competition between CBF1-phyA/B interaction vs PIF4/5-phyB interaction. They have shown this in yeast 3-hybrid assay and also split LUC assays Fig. 7D,E), but in vivo data would strongly support this claim.

Response:

We thank this reviewer for this great suggestion. In response, we performed *in vivo* competition assay using *Arabidopsis* protoplasts, but unfortunately it was not successful. Therefore, we performed semi-*in vivo* pull-down assays using GST-PIF4/PIF5 and His-CBF1 fusion proteins expressed in *E.coli*, and total proteins extracted from 4-d-old phyB-GFP seedlings grown at 22°C in continuous R light. Our data, shown in Fig 5E and F of our revised manuscript, indicated that increasing amounts of His-CBF1 fusion proteins progressively decreased the amounts of GST-PIF4 or GST-PIF5 coprecipitated with phyB-GFP, thus demonstrating that CBF1 indeed inhibited phyB interaction with PIF4 and PIF5.

It is not clear whether the reduction in PIF4/5 level in cbf1 mutant (Fig. 7A) is due to reduced transcriptional induction of PIF4/5 in cbf1 and/or increased degradation of PIF4/5 in the cbf1 mutant background.

Response:

We thank this reviewer for pointing out the incomplete interpretation of our data in our previous manuscript. Our data showed that CBF1 positively regulates *PIF4* and *PIF5* expression in the light by directly binding to their promoters (Fig EV3), and that CBF1 enhanced the stability of PIF4 and PIF5 proteins by inhibiting their interaction with phyB (Fig 5). Therefore, CBF1 promotes PIF4 and PIF5 protein accumulation in the light through both transcriptional and post-translational regulatory mechanisms, and the reduced levels of PIF4/PIF5 proteins in *cbf1* mutants was likely due to both reduced induction of *PIF4/PIF5* transcripts and increased degradation of PIF4/PIF5 proteins. We have revised our manuscript to better interpret our data and summarize our findings.

3. The authors should look into cotyledon area for these mutants as opposite regulation of hypocotyl vs cotyledon area is an indication of hypersensitive phenotype. Shorter hypocotyl might be due to delayed germination or hormone defect or general sickness. This should be ruled out.

Response:

We thank this reviewer for these great suggestions and comments. In response, we examined the germination rates and cotyledon areas of Col, two *cbf1* mutants (*cbf1-1* and *cbf1-2*) and two CBF1-OE lines (*CBF1-myc* and *CBF1-flag*) grown at 22°C in continuous white light. The data, shown in Appendix Fig S1A of our revised manuscript, indicated that the short-hypocotyl phenotype of *cbf1* mutants in the light was not due to delayed seed germination. In addition, the cotyledon areas of CBF1-OE lines were significantly larger than those of Col, whereas *cbf1* mutants did not display any detectable changes in cotyledon areas (Appendix Fig S1B and C). Therefore, we concluded that CBF1 promoted the growth of hypocotyls in the light, while its role in regulating cotyledon expansion needs to be further characterized. Accordingly, we have revised our manuscript to emphasize the regulation of hypocotyl growth by CBF1.

For possible changes in hormone signaling which finally caused the shorter hypocotyls of the *cbf1* mutants, we think that this is reasonable because light triggered changes in plant growth and development are typically mediated by plant hormones (de Wit *et al*, 2016). In addition, because the *cbf1* mutants displayed shorter hypocotyls while the CBF1-OE lines exhibited longer hypocotyls in all tested light conditions but not in darkness (Fig 1), we concluded that the phenotypes were regulated by light but not caused by general sickness of the seedlings.

We strongly agree with this reviewer that light promotes the expansion of cotyledons but inhibits the elongation of hypocotyls. However, this does not necessarily mean that every mutant or OE line should exhibit opposite changes of cotyledon expansion and hypocotyl elongation simultaneously. For example, *35S:SAUR50-GFP* and *35S:SAUR65-GFP* seedlings displayed longer hypocotyls and larger cotyledon areas in the light, whereas *saur50 saur16* mutants developed shorter hypocotyls and smaller cotyledons (Sun *et al*, 2016). In addition, *pif4* and *pif7* mutants developed shorter hypocotyls, but did not display any detectable changes in cotyledon areas in red light (Josse *et al*, 2011). Other examples can be

found in many other studies (Nakamura *et al*, 2006; Mallappa *et al*, 2008; Wu and Spalding, 2007).

4. The expression analysis is strange as shown in Fig. 2C. For example, FR is responding at 3 hr quite strongly compared to other time points, R light is inducing expression at 12 hrs strongly, but not at other time points.

Response:

We have repeated the assays many times, and the induction patterns of *CBF1* expression under different light regimes were very reproducible. It should also be noted that the induction patterns of *CBF1* expression (Fig 2C) in Col correlated well with those of CBF1-myc proteins in *35S:CBF1-myc* under different light regimes (Fig 2F-J). These data indicated that *CBF1* transcript and protein levels were controlled by distinct mechanisms, respectively, under different colors of light, although the underlying molecular mechanisms remain currently unknown.

5. Regardless of the expression of CBF1, the protein is unstable in darkness and stabilized under light. This is a hallmark of COP1-regulated response. They should test whether CBF1 is regulated by COP1 post-translationally. If this is the case, cop1 should display freezing-related phenotype. Previously, it was shown that COP1 is absent in the nucleus at 4C (PNAS), perhaps this allows CBF1 to accumulate at 4C and respond to cold. This is related to the broader picture as they described in the introduction that warm temperature is associated with light and cold is associated with darkness. If CBF1 is unstable in darkness (maybe in cold as well?), how does it respond to cold temperature? There must be a mechanism to stabilize it under cold and dark conditions. Exclusion of COP1 from nucleus at 4C makes sense to stabilize CBF1 and thereby respond to cold. This hypothesis can be tested easily.

Response:

We thank this reviewer for these great questions. In response, we performed *in vitro* pull-down assays, LCI assays, and yeast two-hybrid assays to investigate whether COP1 could physically interact with CBF1. However, the data of all these assays indicated that COP1 and CBF1 could not interact with each other (Fig R2A-C). In addition, we extracted the nuclear proteins from 4-d-old wild-type (Col) and *cop1-4* seedlings grown at 22°C in darkness or continuous W light, and our immunoblot data indicated that there was no obvious difference in the levels of CBF1 proteins in Col and *cop1-4* mutants in both light and dark conditions (Fig R2D). *In vitro* cell-free assays were also performed to compare the degradation of His-CBF1 in the total proteins extracted from 4-d-old light-grown Col and *cop1-4* seedlings, and the data again showed that COP1 did not seem to regulate the degradation of CBF1 proteins (Fig R2E).

Based on these data, it seems likely that COP1 may not be involved in regulating CBF1 stability in darkness at 22°C. The relationship between COP1 and CBF1 at 4°C is obviously beyond the scope of our current study, and will be further investigated in future studies.

Figures for Referees not shown

6. The authors should discuss previous data in the context of their findings. For example, CBF1-PIF4/5 is forming a negative feedback loop where PIFs repress CBF1 and CBF1 activates PIFs. Perhaps this might fit into the discussion about light and cold responses.

Response:

We thank this reviewer for this great suggestion. In response, we discussed the suggested point as follows:

Interestingly, PIF3, PIF4 and PIF7 were shown to directly bind to the *CBF* gene promoters and repress their expression under low temperatures (Kidokoro *et al*, 2009; Lee and Thomashow, 2012; Jiang *et al*, 2017). Thus, CBF1 and PIFs form a negative feedback loop where CBF1 activates *PIFs* but PIFs repress *CBF1*. It will be fascinating to explore how this regulatory loop is modulated under different temperatures.

The discussion of the manuscript is too long (almost 4 pages). They can cut back and discuss broader pictures of general interest and the significance of their study.

Response:

We thank this reviewer for this great suggestion. In response, we have rewritten our Discussion and tried our best to discuss a broader picture of general interest and significance for our study.

7. Figure 4 shows genetic interaction with phy mutants. The data show that cbf1 suppresses phyA and phyB mutant phenotype, suggesting that CBF1 doesn't function in phy signaling pathway. This makes sense if it is functioning in another pathway and attenuating phy signaling pathway.

Response:

We thank this reviewer for asking this great question. We explained the phenotypes of *cbf1-1 phyA-211* and *cbf1-1 phyB-9* double mutants in our revised manuscript as follows:

Firstly, our immunoblots showed that the level of PIF4 in *cbf1-1 phyB-9* mutants was higher than in Col, but lower than in *phyB* mutants in continuous W and R light (Fig EV4A and B). PIF4 also accumulated in a similar pattern in Col, *phyA-211*, and *cbf1-1 phyA-211* mutants grown in continuous FR light (Fig EV4C). Considering the pivotal role of PIF4 in integrating light and temperature control of hypocotyl growth (Nusinow *et al*, 2011; Kumar *et al*, 2012; Sun *et al*, 2012; Jung *et al*, 2016; Quint *et al*, 2016), the steady-state levels of endogenous PIF4 proteins correlated with the hypocotyl lengths of the respective seedlings grown in the light.

Secondly, it was previously reported that *phyB pif4 pif5* mutants and *phyB pifq* mutants also displayed intermediate hypocotyl phenotypes under high R/FR light (Lorrain *et al*, 2008) and continuous R and W light (Leivar *et al*, 2012), respectively. The phenotypes were explained by a mutually negative regulatory loop between phyB and PIFs (Leivar *et al*, 2012). Thus, similar phenotypes of light-grown *cbf1 phyB*, *phyB pif4 pif5* and *phyB pifq* mutants further reinforce the

notion that CBF1 and PIF4/PIF5 play similarly important roles in regulating hypocotyl elongation in the light.

Our data showed that CBF1 physically interacted with phyA and phyB (Fig 3), *CBF1* transcript and protein levels were induced by phyA and phyB in R light (Fig EV1), and CBF1 promoted PIF4 and PIF5 protein accumulation in the light by directly binding to their promoters to induce their gene expression (Fig EV3) and by inhibiting their interaction with phyB (Fig 5). Therefore, our study demonstrated that CBF1 functions in the phytochrome signaling pathway, and its role is closely tied to the phytochrome-PIF signaling module.

8. Fig. 6C should be done in dark vs light conditions for PIF4/5 expression.

Response:

We thank this reviewer for this good suggestion. In response, we examined the expression levels of *PIF4*, *PIF5*, *PIF1* and *PIF3* in 4-d-old Col, *cbf1-1*, *cbf1-2* and *CBF1-myc* seedlings grown at 22°C in darkness. The data, shown in Appendix Fig S9 of our revised manuscript, indicated that CBF1 regulation of *PIF4* and *PIF5* expression predominates in the light relative to the dark.

Referee #3:

In this manuscript, the authors investigated the potential roles of CBF1, an important regulator of cold responses, during photomorphogenesis. They observed that CBF1 activates the expression of PIF4 and PIF5 genes by directly binding to the gene promoters. Moreover, they found that CBF1 interacts directly with phyB, resulting in the enhancement of the stability of PIF4 and PIF5 proteins. In conjunction with the photomorphogenic hypocotyl growth phenotypes of CBF1-deficient mutants and double mutants lacking both CBF1 and phyB, they concluded that CBF1 integrates light and temperature signals to modulate photomorphogenic hypocotyl growth. The biochemical data are strong and well-presented. While the topic is interesting in the field, the authors need to address some critical points, such as the interpretation of molecular genetic data and the effects of cold temperatures on photomorphogenesis to solidify the conclusion.

Major concerns:

1. Figure 4: Both the cbf1-1 phyA-211 and cbf1-1 phyB-9 double mutants exhibited intermediate hypocotyl growth phenotypes compared to those of individual single mutants, obscuring the functional linkage between CBF1 and phytochromes during photomorphogenesis. In addition, the hypocotyl phenotypes of the single and double mutants are not strong enough to support the role of CBF1 in phytochrome-mediated photomorphogenesis (see hypocotyl phenotypes of less than 10% differences in Figure 1). The authors need to clearly address the ambiguous genetic relationship between CBF1 and phytochromes.

Response:

We thank this reviewer for asking this great question. We explained the phenotypes of *cbf1-1 phyA-211* and *cbf1-1 phyB-9* double mutants in our revised manuscript as follows:

Firstly, our immunoblots showed that the level of PIF4 in *cbf1-1 phyB-9* mutants was higher than in Col, but lower than in *phyB* mutants in continuous W and R light (Fig EV4A and B). PIF4 also accumulated in a similar pattern in Col, *phyA-211*, and *cbf1-1 phyA-211* mutants grown in continuous FR light (Fig EV4C).

Considering the pivotal role of PIF4 in integrating light and temperature control of hypocotyl growth (Nusinow *et al*, 2011; Kumar *et al*, 2012; Sun *et al*, 2012; Jung *et al*, 2016; Quint *et al*, 2016), the steady-state levels of endogenous PIF4 proteins correlated with the hypocotyl lengths of the respective seedlings grown in the light.

Secondly, it was previously reported that *phyB pif4 pif5* mutants and *phyB pifq* mutants also displayed intermediate hypocotyl phenotypes under high R/FR light (Lorrain *et al*, 2008) and continuous R and W light (Leivar *et al*, 2012), respectively. The phenotypes were explained by a mutually negative regulatory loop between phyB and PIFs (Leivar *et al*, 2012). Thus, similar phenotypes of light-grown *cbf1 phyB*, *phyB pif4 pif5* and *phyB pifq* mutants further reinforce the notion that CBF1 and PIF4/PIF5 play similarly important roles in regulating hypocotyl elongation in the light.

Our data showed that CBF1 physically interacted with phyA and phyB (Fig 3), *CBF1* transcript and protein levels were induced by phyA and phyB in R light (Fig EV1), and CBF1 promoted PIF4 and PIF5 protein accumulation in the light by directly binding to their promoters to induce their gene expression (Fig EV3) and by inhibiting their interaction with phyB (Fig 5). Therefore, our study demonstrated that CBF1 functions in the phytochrome signaling pathway, and its role is closely tied to the phytochrome-PIF signaling module.

2. Figure 7A: It has been reported in multiple references that PIF proteins are rapidly degraded in the light, which is critical for the induction of photomorphogenesis. The authors found that the levels of PIF4 proteins in Col-0 seedlings were increased in white light or red light conditions compared to those under dark conditions (Figure 7A). The authors need to address this inconsistency.

Response:

We thank this reviewer for asking this great question. Firstly, we performed light shift assays by transferring 4-d-old etiolated Col seedlings to R light for different times. Our immunoblot data showed that indeed, endogenous PIF3, PIF4 and PIF5 proteins were rapidly degraded upon R light exposure (Fig R3), consistent with previous reports (Al-Sady *et al*, 2006; Nozue *et al*, 2007; Shen *et al*, 2007; Lorrain *et al*, 2008). These data indicated that there was no problem with our assay system.

Secondly, we hope to emphasize that we examined the steady-state levels of endogenous PIF4 and PIF5 proteins in 4-d-old Col, *cbf1* mutants (*cbf1-1* and *cbf1-2*), and *CBF1-myc* seedlings grown in darkness or continuous W or R light. Our data showed that the steady-state levels of PIF4 and PIF5 proteins were higher in continuous W or R light compared to those in the dark (Fig 4A of our revised manuscript). Notably, it was also observed in a recent study that higher levels of PIF4 accumulated in Col seedlings in continuous R light than in darkness (Park *et al*, 2018), consistent with our data.

Thirdly, a previous study using 35S:PIF4-HA and 35S:PIF5-HA lines observed the re-accumulation of PIF4 and PIF5 proteins under prolonged R light exposure (Lorrain *et al*, 2008), suggesting that the levels of PIF4 and PIF5 proteins were up-regulated under prolonged R light irradiation.

Therefore, our data, together with those reported in previous studies, indicated that the levels of PIF4 and PIF5 proteins rapidly decreased upon R light exposure; however, under prolonged R light irradiation or in continuous R light, phytochromes induce CBF1 accumulation in the light, which in turn promotes PIF4 and PIF5 protein accumulation. We hypothesize in our revised manuscript that this

dual regulation of PIF4 and PIF5 by phytochromes could prevent plants from over-responding to prolonged light exposure.

Figures for Referees not shown

They also need to show quantification of all western data in the figures.
Response:
We thank this reviewer for this great suggestion. In response, we have quantified our western data in our revised manuscript.

3. The authors concluded that CBF1 integrates cold temperature and light signals into photomorphogenesis. However, no direct experiments on the relationship between cold and light were conducted in this study. Are the photomorphogenic responses influenced by cold temperature treatments?
Response:
We thank this reviewer for asking this great question. In response, we examined the hypocotyl growth of Col seedlings grown under LD conditions at 22°C, 17°C, and 4°C, and observed that the hypocotyl growth was moderately inhibited at 17°C, but severely suppressed at 4°C (Fig 6A and B of our revised manuscript). These observations indicated that low temperatures inhibited *Arabidopsis* hypocotyl growth.

Does the CBF1-medaited regulation of PIF4 and PIF5 play roles during photomorphogenesis at cold temperatures?
Response:
We thank this reviewer for asking this great question. In response, we grew Col, two *cbf1* mutants and two CBF1-OE lines under LD conditions at 22°C, 17°C, and 4°C. After measuring the ratios of hypocotyl lengths at 17°C versus 22°C for the respective genotypes, we found that compared with Col, the *cbf1* mutants displayed increased sensitivity, whereas the CBF1-OE lines exhibited decreased sensitivity to hypocotyl growth inhibition at 17°C (Fig 6C). However, Col, *cbf1* mutants and CBF1-OE lines displayed similar hypocotyl growth inhibition at 4°C (Fig 6A and B). These data suggested that CBF1 attenuated hypocotyl growth inhibition caused by the lower temperature.

In addition, our immunoblot data indicated that PIF4 proteins accumulated to higher levels in Col seedlings grown at both 17°C and 4°C than at 22˚C (Fig 6E). However, the levels of PIF4 proteins were comparable at 22°C and at 17°C in the two *cbf1* mutant seedlings (Fig 6E), indicating that CBF1 was responsible for promoting PIF4 protein accumulation at 17°C. Moreover, we found that PIF4 proteins accumulated to similar levels in *cbf1* mutants and CBF1-OE lines at 4°C (Fig 6E), suggesting that CBF1 may not be involved in regulating PIF4 protein accumulation under cold stress. Similar patterns were also observed for CBF1 regulation of PIF5 protein abundance at 17°C and 4°C (Fig 6F). Together, our data demonstrated that CBF1 promoted PIF4/PIF5 protein accumulation and hypocotyl growth at 22°C and 17°C, but not at 4°C, with a more prominent role at 17°C than at 22°C.

The CBF1 expression is increased by cold temperatures, and CBF1 enhances hypocotyl growth in the light. On the other hand, hypocotyl growth is suppressed at low ambient temperatures (maybe at cold temperatures as well), which is contradictory to the data in this study. It needs to be clarified whether CBF1-mediated photomorphogenic regulation plays a role in integrating light and temperature signals.
Response:

Indeed, our qRT-PCR data showed that the transcript level of *CBF1* was much higher (~15-20 fold) in Col seedlings at 17°C than at 22°C, and even higher at 4°C (Fig 6D). Our data also indicated that the hypocotyl growth of all tested materials was moderately inhibited at 17°C, but severely suppressed at 4°C, and that CBF1 promoted PIF4/PIF5 protein accumulation and hypocotyl growth at 22°C and 17°C, but not at 4°C, with a more predominant role at 17°C than at 22°C (Fig 6). Together, our data demonstrated that CBF1 integrated light and temperature control of hypocotyl growth.

Based on these new data, we also discussed the biological significance of CBF1 in promoting PIF4/PIF5 protein accumulation and hypocotyl growth under ambient temperatures as follows:

Notably, our data showed that CBF1 protein was barely detectable in the dark, but *CBF1* transcript and protein levels were induced in the light, and this induction was mediated by phyA and phyB (Fig 2 and EV1). Therefore, in the light, phytochromes repress hypocotyl growth by inducing phosphorylation and degradation of PIF4 and PIF5 (Nozue *et al*, 2007; Shen *et al*, 2007; Lorrain *et al*, 2008); at the same time, phytochromes induce CBF1 accumulation in the light, which in turn promotes PIF4 and PIF5 protein accumulation (Fig 7). It seems likely that this dual regulation of PIF4 and PIF5 by phytochromes could prevent plants from over-responding to prolonged light exposure.

In addition, our data indicate that CBF1 promotes hypocotyl growth at 22°C and 17°C, with a more prominent role at 17°C than at 22°C (Fig 6). These observations are reminiscent of the fact that *Arabidopsis thaliana*, both winter and summer annual ecotypes, mostly germinate and establish seedlings under relatively low ambient temperatures (spring for summer annuals and fall for winter annuals) (Koornneef *et al*, 2004). We hypothesize that during the dark-to-light transition upon seedlings' emergence from soil, the role of CBF1 may be to maintain proper hypocotyl growth under low ambient temperatures. This may be essential for seedling establishment and vital for survival of plants in changing natural environments. Consistent with this hypothesis, studies of natural *Arabidopsis* populations revealed that in contrast to the various frame-shift mutations or nonsynonymous substitutions found in *CBF2* and *CBF3*, almost no frameshift or premature stop codon has been found in *CBF1* (Kang *et al*, 2013; Monroe *et al*, 2016), suggesting a distinct but pivotal role of CBF1 in plant survival under natural conditions.

Minor comments

1. English grammar would be improved by language editing.

Response:

We thank this reviewer for pointing this out. In response, our manuscript has been edited by an American plant biologist. We believe that the grammar and language of our revised manuscript has been greatly improved.

2. Potential roles of the CBF1-phyB-PIF3/4 in thermotolerance and thermomorphogenesis need to be discussed in more detail.

Response:

We thank this reviewer for this suggestion. In response, the potential role of CBF1 in thermomorphogenesis has been discussed as follows:

Moreover, given the recent finding that phyB is a thermosensor of ambient temperature (Legris *et al*, 2016; Jung *et al*, 2016; Casal and Balasubramanian,

2019), and that PIF4 act as a central regulator of plant thermomorphogenesis (Koini *et al*, 2009; Kumar *et al*, 2012; Quint *et al*, 2016), it will be intriguing to investigate whether CBF1 is also involved in regulating plant thermomorphogenesis.

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2nd Editorial Decision 2nd Apr 2020

Thank you for submitting a revised version of your manuscript. I sincerely apologise for the delay in communicating the decision. I have now received reports from all of the original referees, who find that their main concerns have been addressed and are now in broadly favour of publication of the manuscript. There now remain only a few mainly editorial issues that have to be addressed before I can extend formal acceptance of the manuscript:

REFEREE REPORTS:

Referee #1:

This study describes a differential function of the CBF1 factor, as compared to CBF2 and CBF3, in promoting hypocotyl elongation in the light. CBF1 directly binds the PIF4/ PIF5 promoters and is shown to interact with the phyA and phyB photoreceptors in a competitive manner with PIFs, therefore contributing to the stabilization of these factors in the light. In this revised form, they have addressed most of the concerns issued by the reviewers by providing further in vivo evidence for this regulatory mechanism, in addition to test its relevance under cool temperatures leading to cold-induced activation of CBF1. Notably, these studies showed that CBF1 has a more prominent role in promoting PIF4/PIF5 protein accumulation and hypocotyl elongation at17ºC, than 22ºC or 4ºC. In agreement with the proposed model, cbf1 mutant and OX lines displayed opposite hypocotyl lengths when germinated in the light, but not in the dark, hence further supporting a role of the light receptor phytochromes in this response. However, such a control is not observed at 4ºC, despite CBF1 expression is strongly induced at this temperature. This is indicative of a more complex mechanism of regulation, as suggested by the overlapping DEGs in cbf1 mutants and over-expression lines. This is an unexpected finding that authors barely address in the manuscript. In this regard, although the PIF4 and PIF5 are included in the list of differentially expressed genes, consistent with a role of CBF1 in the transcriptional regulation of these factors, PIF4 and PIF5 targets (i.e. auxin biosynthetic and signaling genes or cell wall remodeling enzymes) are not really over-represented in this dataset. HY5 was initially observed to be misregulated in the CBF1 lines and this might be a relevant finding that would explain these partially inconsistent results. Also, authors report that CBF1 does not physically interact with COP1 and based on these findings it would be important to propose a mechanistic model why which effects on hypocotyl growth of CBF1 regulation of PIF4 and PIF5 levels are more relevant in a narrow temperature range around 17ºC.
Minor points:
Figure 4: western blots in panel A detect the endogenous PIF4 protein and clock regulation of PIF4 transcription may explain lower levels of the protein in darkness. However, in panel C PIF4 is expressed under control of the 35S promoter and levels of the protein are also surprisingly lower than in white or red light.
Figure 6E/F: PIF4 protein levels in Col-0 are higher at 17ºC than 22ºC, while PIF5 levels are increased at 4ºC. This seems to be in controversy with the reported role of elevated ambient temperatures in up-regulating PIF4/PIF5 expression via suppressed repression by the EC. Although seedlings were grown here in LDs and temperature effects on EC function were analyzed in SD, this is a rather surprising finding.
Three hybrid assays: Why were yeast cells selected on -Trp-Leu-His. Was the -His auxotrophy used

for the selection of the CBF1 construct?

Referee #2:

Majority of my concerns have been adequately addressed. This is a much better story now. The claims are supported by experimental evidence.

Referee #3:

The authors peformed the suggested experiments and assays, which significantly contributed to the solidification of the data. They also properly responded to the comments as to the previous and current data in terms of the novel physiological function of CBF1 in the signaling linkage between light and temperature signlas during hypocotyl photomorphogenesis.

One minor issue concerning in the discussion section.The reviewer is concerned about overstatement or generalization of their findings in some discussions. It would be better to tone down the description.

2nd Revision - authors' response 5th Apr 2020

Reviewers' comments:

Referee #1:

This study describes a differential function of the CBF1 factor, as compared to CBF2 and CBF3, in promoting hypocotyl elongation in the light. CBF1 directly binds the PIF4/ PIF5 promoters and is shown to interact with the phyA and phyB photoreceptors in a competitive manner with PIFs, therefore contributing to the stabilization of these factors in the light. In this revised form, they have addressed most of the concerns issued by the reviewers by providing further in vivo evidence for this regulatory mechanism, in addition to test its relevance under cool temperatures leading to cold-induced activation of CBF1. Notably, these studies showed that CBF1 has a more prominent role in promoting PIF4/PIF5 protein accumulation and hypocotyl elongation at17ºC, than 22ºC or 4ºC. In agreement with the proposed model, cbf1 mutant and OX lines displayed opposite hypocotyl lengths when germinated in the light, but not in the dark, hence further supporting a role of the light receptor phytochromes in this response.

However, such a control is not observed at 4ºC, despite CBF1 expression is strongly induced at this temperature. This is indicative of a more complex mechanism of regulation, as suggested by the overlapping DEGs in cbf1 mutants and over-expression lines. This is an unexpected finding that authors barely address in the manuscript. In this regard, although the PIF4 and PIF5 are included in the list of differentially expressed genes, consistent with a role of CBF1 in the transcriptional regulation of these factors, PIF4 and PIF5 targets (i.e. auxin biosynthetic and signaling genes or cell wall remodeling enzymes) are not really over-represented in this dataset. HY5 was initially observed to be misregulated in the CBF1 lines and this might be a relevant finding that would explain these partially inconsistent results. Also, authors report that CBF1 does not physically interact with COP1 and based on these findings it would be important to propose a

mechanistic model why which effects on hypocotyl growth of CBF1 regulation of PIF4 and PIF5 levels are more relevant in a narrow temperature range around 17ºC.

Response:

We thank this reviewer for these great comments. Indeed, the integration mechanisms between light and temperatures have proven to be complicated, and at present we are unable to explain why CBF1 does not regulate PIF4/PIF5 protein accumulation at 4°C, although *CBF1* expression is strongly induced under this temperature. It seems likely that the regulatory mechanisms under ambient temperatures (such as 17°C-22ºC) and cold stress (such as 4°C) are essentially different.

Indeed, *HY5* expression was shown to be decreased in both *cbf1-1* and *CBF1*-overexpression seedlings (Fig S6 of our firstly submitted manuscript). However, this pattern was only prominent for *HY5* but not for the other examined genes (Fig EV3C and S8 of our revised manuscript). The inconsistency of *HY5* expression changes in *cbf1* mutants and *CBF1*-overexpression seedlings could not be explained at present, and will be further investigated in future studies.

For the possible relationship between CBF1 and COP1, we provided evidence in our previous response letter that COP1 did not interact with CBF1 and may not be involved in regulating CBF1 stability in darkness at 22°C. The relationship between COP1 and CBF1 at 4°C is obviously beyond the scope of our current study, and will be further investigated in future studies.

Minor points:

Figure 4: western blots in panel A detect the endogenous PIF4 protein and clock regulation of PIF4 transcription may explain lower levels of the protein in darkness. However, in panel C PIF4 is expressed under control of the 35S promoter and levels of the protein are also surprisingly lower than in white or red light.

Response:

We thank this reviewer for these comments. Indeed, the level of 35S-driven PIF4 was lower in darkness than in white or red light. We repeated the assays many times, and the data were very reproducible. These observations could be explained by our findings that CBF1 accumulates in the light and could promote PIF4 protein abundance post-translationally by inhibiting its interaction with phyB.

Figure 6E/F: PIF4 protein levels in Col-0 are higher at 17ºC than 22ºC, while PIF5 levels are increased at 4ºC. This seems to be in controversy with the reported role of elevated ambient temperatures in up-regulating PIF4/PIF5 expression via suppressed repression by the EC. Although seedlings were grown here in LDs and temperature effects on EC function were analyzed in SD, this is a rather surprising finding.

Response:

We thank this reviewer for these comments. It should be noted that the gene expression changes of *PIF* are often inconsistent with the changes of their protein abundance under diurnal conditions (Leivar and Monte, 2014). In our study, we examined the PIF4 and PIF5 protein levels, while the study mentioned by the reviewer examined the *PIF4* transcript levels (Mizuno *et al*, 2014). In addition, as mentioned by this reviewer, we used LD condition in our study, while the study of Mizuno *et al* (2014) used SD condition. It was previously shown that the expression patterns of *PIF4* and *PIF5* were essentially different under different photoperiodic conditions (Niwa *et al*, 2009). Therefore, our data may not be in

controversy with the study of Mizuno *et al* (2014), and the inconsistency may be due to the different levels examined for PIF4/PIF5 and different experimental conditions used in two studies.

Three hybrid assays: Why were yeast cells selected on -Trp-Leu-His. Was the -His auxotrophy used for the selection of the CBF1 construct?
Response:
Yes, His was the nutritional selection marker for the pRS423 vector. In our yeast three-hybrid assays, three vectors were used: pD153 (to express phyB-BD), pGADT7 (to express AD-PIF4 or AD-PIF5), and pRS423 (modified to express CBF1). The nutritional selection markers in yeast are Trp, Leu and His, respectively, for these three vectors.

Referee #2:

Majority of my concerns have been adequately addressed. This is a much better story now. The claims are supported by experimental evidence.
Response:
We thank this reviewer for the comments and suggestions to improve our manuscript.

Referee #3:

The authors peformed the suggested experiments and assays, which significantly contributed to the solidification of the data. They also properly responded to the comments as to the previous and current data in terms of the novel physiological function of CBF1 in the signaling linkage between light and temperature signlas during hypocotyl photomorphogenesis.
Response:
We thank this reviewer for the comments and suggestions to improve our manuscript.

One minor issue concerning in the discussion section. The reviewer is concerned about overstatement or generalization of their findings in some discussions. It would be better to tone down the description.
Response:
We thank this reviewer for these great comments. In response, we have revised our Discussion to tone down some conclusions. The changes have been highlighted in our revised manuscript.

REFERENCES
Leivar P, Monte E (2014) PIFs: systems integrators in plant development. *Plant Cell* 26: 56–78
Mizuno T, Nomoto Y, Oka H, Kitayama M, Takeuchi A, Tsubouchi M, Yamashino T (2014) Ambient temperature signal feeds into the circadian clock transcriptional circuitry through the EC night-time repressor in *Arabidopsis thaliana*. *Plant Cell Physiol* 55: 958–76

Niwa Y, Yamashino T, Mizuno T (2009) The circadian clock regulates the photoperiodic response of hypocotyl elongation through a coincidence mechanism in *Arabidopsis thaliana*. *Plant Cell Physiol* 50: 838–54

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jigang Li
Journal Submitted to: THE EMBO JOURNAL
Manuscript Number: EMBOJ-2019-103630

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	To analyze the differences in the wild type, cbf1 mutants and CBF1-overexpression lines at different temperatures, we measured two plates at the same temperature in one biological experiment. Each plate has about 30 seedlings for wild type, cbf1 mutant and CBF1-overexpression lines, respectively. To examine gene expression or protein abundance, RNAs or proteins were extracted from about 0.15 g of seedlings grown on the same plate for the same treatment time in one biological experiment.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable, no animals were used in that study.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis.
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.	No specific step was taken.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
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.	No specific step was taken, but immunofluorescence samples were quantified using the imageJ software.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Student's t-test (two tailed) and one way ANOVA were used to analyze significant differences.
Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes

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).	Mouse monoclonal anti-myc (Mei5 Biotechnology Cat#MF083) Mouse monoclonal anti-His (Sigma-Aldrich Cat#H1029) Rabbit polyclonal anti-GST (Sigma-Aldrich Cat#G7781) Rabbit polyclonal anti-H3 (Abcam Cat#ab1791) Mouse monoclonal anti-HSP (Beijing Protein Innovation Cat#AbM51099-31-PU) Goat polyclonal anti-PIF4 (Agrisera Cat#AS163955) Rabbit polyclonal anti-PIF5 (Agrisera Cat#AS122112) Rabbit polyclonal anti-phyA (Zhang et al., 2018) Rabbit polyclonal anti-RPN6 (Zhou et al., 2018)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Sequence Read Archive PRJNA608253
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).	NA
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).	NA
21. 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.	NA

G- Dual use research of concern

22. 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.	NA