The E3 Ubiquitin Ligase HAF1 Modulates Circadian Accumulation of EARLY FLOWERING3 to Control Heading Date in Rice Under Long-day Conditions

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Review timeline:

TPC2018-00235-RA	Submission received: 1st Decision:	March 22, 2018 May 13, 2018 *manuscript declined*
TPC2018-00653-RA	Submission received: 1st Decision: Final acceptance: Advance publication:	August 28, 2018 Sept. 2, 2018 *acceptance pending, sent to science editor* Sept. 14, 2018 Sept. 21, 2018

REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00235-RA 1st Editorial decision – *declined* **May 13, 2018**

The most critical comments were as follows.

Reviewer 1 requests full-length protein interaction data and the use of an additional method such as BiFC. If you do select the latter method then be sure to use the appropriate controls as described in The Plant Cell by Kudla and Bock (2016). This review points out that the antibody developed needs to be properly controlled and shown in the Supplemental Data, which is essential. Also, clarify contradictions with the protein degradation data, as described, and deal with the doubts about the thoroughness of the natural variation data and its novelty.

Reviewer 2 suggests changing the structure of the manuscript. We do not request this, as it was not requested by the other reviewers who found the structure logical. However, the smaller textual issues raised by this reviewer seem useful.

Reviewer 3 makes an important point about repetition and quantification of the immunoblots. Also, the reviewer asks for the effect of *haf1* on clock gene expression in free-running conditions and this seems an important issue if you wish to reach the conclusion that it does not affect clock-regulated gene expression. Analyzing the OsELF3 protein in selected accessions carrying the different OsELF3 alleles seems worthwhile, and is relatively straightforward with the antibody raised. If you decide to resubmit a revised version, please deal with these and all of the other points raised by the reviewers. Also provide a document describing your response to each point.

--- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00653-RA Submission received **August 28, 2018**

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

This manuscript describes the role of HAF1, a RING-domain ubiquitin ligase, in the regulation of photoperiodic flowering of rice. Previous work from the same lab resulted in the isolation of the *haf1* mutant, which flowers late under both short days (SD) and long days (LD) (Yang et al., 2015). The HAF1 protein was shown to interact with Hd1 protein to promote its ubiquitination and reduce its abundance under SD. This mechanism contributes to modulating the heading of rice under SD, but no mechanism had been described to account for the role of HAF1 under LD. In this work, the authors show that under LD, HAF1 interacts with OsELF3, a homologue of Arabidopsis ELF3, and that the interaction leads to ubiquitination and proteasome-mediated degradation of OsELF3. Polymorphisms in the OsELF3 sequence that alter the interaction domain of OsELF3 show a latitudinal distribution, and are discussed as required for geographic adaptation.

The authors address relevant aspects of rice reproductive development. The question of how Hd1 switches from an LD-repressor to an SD-activator of flowering is an important biological problem that is still unresolved, and HAF1 might be a component of such switching mechanism. Additionally, the role of natural variation and artificial selection in the process of rice latitudinal expansion is being addressed through the analysis of OsELF3, adding an applied facet to the study. Some points need to be clarified though, before concluding on both aspects.

Point 1. The authors start the Results section by exploring the physical interaction between HAF1 and OsELF3, but no explanation is given on how this hypothesis was first developed. Why was this interaction tested in the first place?

RESPONSE: We have explained this in the last paragraph in the Introduction. Our previous investigation suggested that the *haf1 hd1* double mutant flowers as late as *haf1* and that HAF1 may interact with additional components to control the heading date under long-day conditions (Yang et al., 2015). In this study, we try to examine the possible interaction between HAF1 and some flowering regulators under LDs, such as Ghd7, Ghd8, Ghd7.1, Ehd4 and OsELF3. Finally, we characterize OsELF3, a flowering promoter under LDs, which physically interacts with HAF1.

Point 2. Yeast-2-hybrid data indicate an interaction between HAF1 and OsELF3, however, only truncated versions of HAF1 were used. The data should also include a HAF1 full-length control. If this was excluded because of auto-activation, a domain swap between AD and BD might be performed. Also, it should be explained why these specific fragments were selected in this experiment. Do they correspond to functional sub-domains of the HAF1 protein? I could not find this info in Yang et al. 2015 either. Finally, BiFC experiments should be done also, including a OsELF3 (G) control.

RESPONSE: Due to the auto transcriptional activation activity of HAF1, we have taken your suggestion and performed yeast two-hybrid assays with full-length HAF1 protein as prey. Yeast two-hybrid assays showed that constructs containing the full-length HAF1 protein indeed interact with OsELF3 (Figure 1A). Considering that the C3HC4 domain exists in the C-terminal region of HAF1 (Yang et al., 2015), the interaction with OsELF3 might occur through C3HC4 domain of HAF1. In the BiFC experiments, the construct c-YFP-OsELF3 (L558S) harboring the substitution of leucine to serine in residue 558 (L558S) at the C terminus of OsELF3 abolished the interaction with HAF1 (Figure 2A).

Point 3. Analysis of OsELF3 protein by western blot. The authors have developed an antibody against OsELF3 and detection was performed on OsELF3 purified from *E. coli*, as well as on total protein extracts from rice leaves. Based on these data, the authors conclude that the antibody "specifically detects OsELF3". This is not correct because a control of specificity is missing. Total extracts from rice leaves should be done also alongside an *oself3* KO mutant, to exclude the possibility that the antibody might be non-specific. The antibody was indeed used on a diurnal time course (Fig. 4G) of *oself3* mutants, but this mutation is not abolishing OsELF3 protein expression.

RESPONSE: In this version, we have checked the specificity of the OsELF3 antibody using total protein extracts from wild-type (WT) variety ‘Zhonghua 11 (ZH11) or ‘Zhenshan 97’ (ZS97) leaves as samples; a specific band was also detected (Supplemental Figure 1E, F). In the sample from a knock-down mutant of *OsELF3* (Yang et al., 2013), the intensity of the immunoblot band was weak (Supplemental Figure 1F). This result suggested that the OsELF3 antibody specifically detects OsELF3.

Our previous study reported that *oself3* was a knock-down mutant, in which the diurnal expression pattern of *OsELF3* was shifted (Yang et al., 2013). In this study, OsELF3 abundance was detected by anti-OsELF3 antibody. We found that the phase of protein expression pattern rhythm was shift backward in *oself3* (Figure 4G).

Point 4. HAF1-mediated degradation of OsELF3. The experiments presented are not convincingly showing that this is the case. In vitro data of Figure 3D show a slight increase in protein stability of OsELF3 when incubated with protein extracts from *haf1* mutant plants. The difference is in the order of a few minutes, which raises the question of how this is biologically meaningful. But the major issue relates to the MG132 control that should inhibit the proteasome. Yet, in the corresponding gel, MG132 shows very limited capacity to block OsELF3 degradation. Perhaps an additional control including MBP-OsELF3 alone incubated in the same time course could help to exclude that the OsELF3 protein is intrinsically unstable in these conditions.

RESPONSE: We have done more than three biological repeats and found the same results of protein stability of OsELF3 when incubated with protein extracts from *haf1* mutant plants. The UPS pathway is one of the main systems for protein degradation, although in the presence of MG132, protein can be degraded slowly. Other studies have also shown that the target proteins can be slowly degraded in the presence of MG132, such as Fig 5C in “Degradation of monoCuLm 1 by APC/CTAD1 regulates rice tillering”, Fig 6E in “COP1 and ELF3 Control Circadian Function and Photoperiodic Flowering by Regulating GI Stability”. Furthermore, a protein can be regulated by various E3 ubiquitin ligases. For example, Arabidopsis CO protein can be degraded by COP1 in darkness. However, during the daytime, another E3 ubiquitin ligase, HOS1, is required to degrade CO. Our results indicate that OsELF3 could be degraded by the other unknown process, not merely by HAF1-mediated ubiquitin degradation.

We have taken your suggestion and provided an additional control including MBP-OsELF3 alone incubated in the same time course (Figure 3D). The result showed that MBP-OsELF3 protein was stable.

Point 5. The genetics presented in Fig. 6 also disagree with HAF1 degrading OsELF3. In *haf1* mutants, increased OsELF3 levels should promote flowering under LD, but *haf1* mutants are late heading. It might still be possible that a mild increase of OsELF3 represses flowering, whereas strong over-expression causes a dominant negative effect and promotes it. Alternatively, the shift in OsELF3 peak observed in *haf1* mutants (and not its overall abundance) is responsible for the observed phenotypes. In general, it is difficult to reconcile all experimental data.

RESPONSE: We have taken your suggestion and modified our statement. In this version, we generated F1 plant by crossing *OsELF3-OX* plant with the *haf1* mutant. Under natural LDs in Wuhan, the F1 plants exhibited an early heading date similar to *OsELF3-OX* lines (Supplemental Figure 6). Thus, we speculated that the delay heading date of *haf1 oself3* under LDs might be caused mainly by the disruption of diurnal expression pattern in *oself3*, not merely OsELF3 abundance. In addition, the possibility that some flowering repressors are degraded by HAF1 under LDs to contribute to the delayed heading date in *haf1 oself3* plants cannot be excluded.

Point 6. OsELF3 (G/A) variants and geographical distribution of rice varieties. There is certainly a correlation between specific alleles and latitudes, but to demonstrate that OsELF3 variants are causal to this distribution requires further genetic demonstration (e.g. NILs or transgenics). How variable are other flowering time regulators in the collection (e.g. see Gao et al., 2014)? How many accessions are represented by the different haplotypes? Was the SG loss-of-function allele a minor one in the collection (<5%)? Also, this section is not very novel, Matsubara et al., 2012 had already come to similar conclusions based upon mapping the Hd17/OsELF3 QTL.

RESPONSE: Thanks for your comments. The previous study has reported that OsELF3 (G/A) variants caused the amino acid change (Serine to Leucine) in ‘Nipponbare’, and the ‘Nipponbare’ (with OSELF3-L) showed early flowering than its near isogenic lines, NIL- *Hd17 /OsELF3* (Matsubara et al., 2012). These results indicated that variation of *OsELF3 (G/A)* indeed contributes to heading date in rice. Of course, further studies of transgenic lines should be conducted.

We performed GWAS for heading date and found that the second SNP G/A at position 5191 was significantly associated with the heading date ($P = 4.95\times10^{-8}$; Supplemental Figure 8). We also observed the correlation between specific alleles of the SNP and latitudes, suggesting that OsELF3 may be a footprint of natural and artificial selection for geographical distribution during rice breeding. We could not claim that OsELF3 variants are causal for this distribution. Actually, using the same population, a previous study demonstrated that combinations of the *Ghd7*, *Ghd8* and *Hd1* genes largely define the ecogeographical adaptation (Zhang et al., New Phytologist, 2015, 208:1056-66). Using a different population, Gao et al. (2014) claimed that the functional differences at the *DTH7*,

***Ghd7*, or *DTH8* individual locus could partially explain the their contributions to geographical distribution.**

The accession number of each haplotype has been indicated in Supplemental Figure 7B. Only 8 accessions were found to carry the loss-of-function allele (SG). The SG allele is a minor one in the collection (<5%).

Although a previous study found that the OsELF3 (G/A) variants might have been selected in rice during domestication (Matsubara et al., 2012), the molecular interaction of OsELF3 (G/A) variants has not been reported. In this study, our investigation demonstrated that natural variation in the interaction domain of OsELF3 with HAF1 might contribute to the geographic distribution of different rice varieties. Yeast two-hybrid and pull-down assays demonstrated that HAF1 interacted with the OsELF3(L) type, but not interact with the OsELF3(S) type (Figure 1C and Figure 2C). We also prepared total protein extracts from ZS97 leaves to incubate with purified MBP-OsELF3(S) for cell-free degradation assays (Supplemental Figure 9). The protein degradation of MBP-OsELF3(S) showed similar trends when it was incubated with total protein extracts from ZS97 or *haf1* leaves (Supplemental Figure 9A). Thus, our results suggested that HAF1 is only responsible for the ubiquitination and degradation of OsELF3(L), but not for the ubiquitination of OsELF3(S).

Reviewer #2:

In this work, the authors examine HAf1-ELF3 in rice to understand their biochemical and genetic relationships. Using yeast two hybrid assays, the C-terminus of ELF3 was found to associate to a region of HAF1. BiFC and in vitro pull downs supported this. In vitro HAF1 could function as an E3 ligase to ubiquitinate ELF3. A tobacco assay supported this notion. Evening expression of GI was seen in *haf1* plants, and this was phase shifted in *elf3 haf1* plants. The *haf1* mutant is not obviously a clock mutant. In general, ELF3 was epistatic to HAF1 for molecular markers and flowering time under long-day conditions. A geographical association of ELF3 haplotypes was tagged on the story. It was connected back to some of the pull-down work.

Point 1. This work has an inverted narrative I found difficult to follow. Also, the haplotype work at ELF3 is not fully connected to HAF1. It would require extensive revisions to add that, as what is really needed is either i) a repeat of Figure 3 and 4 with both ELF3-A and ELF3-G or better ii) epistasis of ELF3-A with *haf1* mutations vs. ELF3-G with *haf1* mutations. I cannot recommend further revisions of this work until the two stories are more fully connected.

Natural variation in ELF3 in rice has been reported (e.g. Plant Cell Physiol. 2012 Apr;53(4):709-16). This is Hd17 after all. Maybe a story around understanding the haplotypes at ELF3 as being HAF1 dependent is a better narrative to follow, meaning an inversion of the figures and logic. The paper needs extensive editing.

RESPONSE: Thanks for your comments. Our previous examination revealed that HAF1, a C3HC4 RING domain-containing E3 ubiquitin ligase, is essential to precisely modulate the timing of Hd1 accumulation and to ensure an appropriate photoperiodic response under SDs (Yang et al., 2015). Under LDs, the *haf1 hd1* double mutant flowers as late as *haf1*, indicating that HAF1 may interact with additional components to control the heading date under LD conditions (Yang et al., 2015). In this study, we characterize OsELF3, a flowering promoter under LDs, which physically interacts with HAF1. We further confirmed that HAF1 mediates ubiquitination of OsELF3 protein via the 26S proteasome-dependent pathway. Finally, we found that the natural variation in the interaction domain of OsELF3 with HAF1 contributes to the geographic distribution of the *Japonica* rice varieties. Our manuscript is well-organized and the structure is logical. Please reconsider our manuscript.

Point 2. Much of the paragraph lines 109-126 is not relevant to this work. This is not a GI-CO story.

RESPONSE: In this paragraph, we described the research progress on ubiquitin-mediated protein degradation in photoperiodic flowering in Arabidopsis. Although multiple E3 ubiquitin ligases involved in flowering have been characterized in *Arabidopsis*, only a few cases has been studied in rice.

Point 3. The paragraph from line 130 and the results from line 144: there needs to be a better logical argument as to why the authors are looking at a HAF1-ELF3 physical protein-protein interaction.

RESPONSE: We have explained this the last paragraph of the Introduction. Our previous investigation suggested that the *haf1 hd1* double mutant flowers as late as *haf1* and that HAF1 may interact with additional components to control the heading date under long-day conditions (Yang et al., 2015). In this study, we try to examine the possible interaction of HAF1 with some flowering regulators under LDs, such as Ghd7, Ghd8, Ghd7.1, Ehd4 and OsELF3.

Finally, we characterize OsELF3, a flowering promoter under LDs, which physically interacts with HAF1. We have explained this the last paragraph in the Introduction.

Reviewer #3:

In the manuscript titled "HAF1 Modulates Circadian Accumulation of OsELF3 Controlling Heading Date Under Long-day Conditions in Rice" Zhu and colleagues investigate the role of the E3 ubiquitin ligase, HAF1, in controlling the ubiquitination and stability of the circadian clock protein ELF3. To do this, they test interactions between HAF1 and ELF3 using in vitro and in vivo assays. Next, they determine if HAF1 can mediate ubiquitination of ELF3 using in vitro purified proteins. They then study the effects of mutating HAF1 on the stability of ELF3 using cell-free degradation assays and time course western blot analyses. Finally, they analyze various rice accessions for the presence of a mutation in ELF3 that prevents interaction with HAF1. From these studies, they conclude that ELF3 is a substrate of HAF1 and that domestication across latitudinal boundaries is associated with disruption of the HAF1 ELF3 interaction. The work presented here supports the conclusions of the authors and is clearly presented. They use a wide range of complementary techniques to demonstrate that ELF3 is a substrate of HAF1, and the addition of a study looking at the geographical distributions of ELF3 mutants provided context to their molecular, genetic, and biochemical studies.

Point 1. For ELF3 protein measurements (cell-free degradation assays and time course western blot), triplicate repeat and quantitation is necessary to make this convincing. Furthermore, they should cross-load a sample from a single time point (i.e. ZT 0) from each mutant and wild-type time course on the other blots so that cross comparison of protein levels between mutants could be made.

RESPONSE: This is a good suggestion. We have provided the data in Supplemental Figure 2 in this version.

Point 2. It is interesting that the *haf1* mutant has no effect on the expression of clock genes despite having an effect on ELF3 protein levels. Expression of clock genes should be studied in the *haf1*, *elf3*, and *haf1/elf3* mutants in a constant light time course where the circadian clock is free-running. This would demonstrate if HAF1 is regulating clock function, which can't be determined in a diurnal time course.

RESPONSE: Yes, we have taken your suggestion and examined the free-running rhythms of the clock-associated genes under continuous light conditions (Supplemental Figure 4). These results demonstrated that *OsELF3* is required for the rhythmic expression of circadian clock genes. We proposed that HAF1 affects the function of *OsELF3* mainly at the protein level, but not on the diurnal transcription pattern.

Point 3. The results showing association of an ELF3 mutation with domestication were quite interesting, but one further experiment would increase the confidence in this result. Time course western blotting with the ELF3 antibody should be performed on accessions with the ELF3-G and ELF3-A variants to determine the effects on ELF3 protein levels.

RESPONSE: Yes, natural variation in the interaction domain of OsELF3 with HAF1 could be selected during domestication in rice. Yeast two-hybrid and pull-down assay demonstrated that HAF1 interacted with the OsELF3(L) type, but did not interact with that OsELF3(S) type (Figure 1C and Figure 2C).

We have taken your suggestion and done the time course western blotting with the OsELF3 antibody on ZH11 (with OSELF3-L) and ZS97 (with OSELF3-S). In ZS97 leaves, OsELF3 showed a similar diurnal rhythm to that in ZH11, but a relatively lower amount (Figure 4I). We also prepared total protein extracts from ZS97 leaves to incubate with purified MBP-OsELF3(S) for cell-free degradation assays (Supplemental Figure 9). The protein degradation MBP-OsELF3(S) showed similar trends when it was incubated with total protein extracts from ZS97 or *haf1* leaves (Supplemental Figure 9A). Thus, our results suggested that HAF1 is only responsible for the ubiquitination and degradation of OsELF3(L), but not for the ubiquitination of OsELF3(S).

Point 4. Discovering posttranslational regulatory mechanisms for clock proteins has wide importance, and the major conclusions of the manuscript are mostly supported by the data. The suggested experiments would help increase confidence in critical areas of the manuscript.

RESPONSE: Many thanks for your suggestion.

TPC2018-00653-RA 1st Editorial decision – *acceptance pending* **Sept. 2, 2018**

Thank you for the revised version of your manuscript and for including the data and edits to the text requested by the reviewers. We are pleased to inform you that your paper entitled "HAF1 Modulates Circadian Accumulation of OsELF3 Controlling Heading Date Under Long-day Conditions in Rice" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **Sept. 14, 2018**