The RING-Finger Ubiquitin Ligase HAF1 Mediates Heading date 1 Degradation during Photoperiodic Flowering in Rice

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The photoperiodic response is one of the most important factors determining heading date in rice (Oryza sativa). Although rhythmic expression patterns of flowering time genes have been reported to fine-tune the photoperiodic response, posttranscriptional regulation of key flowering regulators has seldom been elucidated in rice. Heading date 1 (Hd1) encodes a zinc finger transcription factor that plays a crucial role in the photoperiodic response, which determines rice regional adaptability. However, little is known about the molecular mechanisms of Hd1 accumulation during the photoperiod response. Here, we identify a C3HC4 RING domain-containing E3 ubiquitin ligase, Heading date Associated Factor 1 (HAF1), which physically interacts with Hd1. HAF1 mediates ubiquitination and targets Hd1 for degradation via the 26S proteasome-dependent pathway. The haf1 mutant exhibits a later flowering heading date under both short-day and long-day conditions. In addition, the haf1 hd1 double mutant headed as late as hd1 plants under short-day conditions but exhibited a heading date similar to haf1 under long-day conditions, thus indicating that HAF1 may determine heading date mainly through Hd1 under short-day conditions. Moreover, high levels of Hd1 accumulate in haf1. Our results suggest that HAF1 is essential to precisely modulate the timing of Hd1 accumulation during the photoperiodic response in rice.

INTRODUCTION

Rice (Oryza sativa) is one of the most important cereal crops in the world. Heading date in rice is a critical determinant of regional adaptability and grain production. Although rice is regarded as a short-day (SD) plant, artificial selection during domestication has developed cultivars adapted to different photoperiods in a broad range of latitudes (Izawa, 2007; Song et al., 2015). The molecular basis for regulation of flowering has been extensively studied using Arabidopsis thaliana and rice, which represent long-day (LD) and SD plants (Song et al., 2015), respectively. Photoperiodic flowering in rice is regulated through two distinct pathways: the GI-Hd1-Hd3a pathway for adaption in SD conditions, a counterpart of the GI-CO-FT pathway in Arabidopsis (Turck et al., 2008); and the unique Ghd7-Ehd1-Hd3a/RFT1 pathway for adaptation in LD conditions (Xue et al., 2008; Tsuji et al., 2011; Song et al., 2015). In contrast to the GI-CO-FT pathway in Arabidopsis, the rice GI-Hd1-Hd3a pathway has evolved a distinct regulation pattern among orthologous pathways in rice. A previous study showed that overexpression of rice GI suppressed Hd3a expression, resulting in late flowering under both SD and LD conditions (Hayama et al., 2003). This result indicated that rice GI functions oppositely to GI in Arabidopsis and suppresses flowering in rice (Fowler et al., 1999; Hayama et al., 2003). Further investigation showed that increased expression of Hd1 coincides with overexpression of GI, suggesting that GI-promoted Hd1 homolog expression is conserved between rice and Arabidopsis (Hayama et al., 2003).

Hd1 is a major quantitative trait locus associated with photoperiod sensitivity among different rice cultivars (Yano et al., 2000). Recently, a genome-wide association study revealed that Hd1 gene variation plays an important role in rice adaptation, which determines the distribution of varieties across low to high latitudes (Zhao et al., 2011; Huang et al., 2012). Allelic variation of Hd1 is involved in SD flowering in rice, suggesting that variation in Hd1 proteins contributes to the diversity of flowering times in cultivated rice (Takahashi et al., 2009). Molecular analyses have demonstrated that Hd1 is a crucial regulator of the photoperiodic flowering pathway in rice (Izawa et al., 2002; Hayama et al., 2003). Although the function of Hd1 in rice is highly similar to that of CO in Arabidopsis, the direct regulation of Hd3a by Hd1 has not been confirmed. Overexpression of Hd1 has been reported to suppress Hd3a and delay flowering under SD conditions (Ishikawa et al., 2011). By contrast, constitutive expression of CO promotes flowering by increasing FT transcription in Arabidopsis (Onouchi et al., 2000). Gene expression analysis has revealed that Hd1 promotes FT-like genes in the inductive SD conditions and represses FT-like gene expression in noninductive LD conditions (Izawa et al., 2002). This antagonistic action of Hd1 activity may be regulated posttranscriptionally because the expression pattern of Hd1 is almost the same in different photoperiods (Izawa et al., 2002; Ishikawa et al., 2011). Hd1 expression is under circadian control, and it peaks at dusk under LD conditions (Yano et al., 2000; Izawa et al., 2002). In the presence of light, the Hd1 protein complex suppresses Hd3a under LD conditions (Izawa et al., 2002; Ishikawa et al., 2011).
Further investigation showed that Hd1 protein levels in Hd1-overexpressing plants are not altered in the presence of light (Ishikawa et al., 2011). Suppression of Hd3a and delayed flowering by overexpression of Hd1 under SD conditions depend on phyB, indicating that light may modulate Hd1 protein control of Hd3a transcription (Ishikawa et al., 2011). In Arabidopsis, posttranslational regulation of CO protein is crucial for the photoperiodic induction of FT expression (Jang et al., 2008). Various light signals modulate CO protein stability throughout the day (Valverde et al., 2004; Jang et al., 2008). Blue light has been shown to stabilize CO, but red light acts to reduce CO abundance in a phyB-dependent manner (Valverde et al., 2004; Jang et al., 2008; Liu et al., 2008). Although previous studies suggest Hd1 promotes flowering under SD conditions and suppresses it under LD conditions (Yano et al., 2000; Izawa et al., 2002; Hayama et al., 2003; Ogiso et al., 2010), the mechanism underlying how Hd1 abundance is regulated has not previously been reported.

The ubiquitin 26S proteasome system (UPS) is critical in enabling plants to alter their proteome to integrate internal and external signals for developmental plasticity and environmental adaptation (Chen and Hellmann, 2013; Wang and Shi, 2014). The UPS pathway, including ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s), catalyzes the ubiquitination of various protein substrates for targeted degradation via the 26S proteasome (Chen and Hellmann, 2013). Substrate specificity mainly depends on the type of E3 ligase, which includes three main classes: HECT, RING, and U-box in plants (Chen and Hellmann, 2013). In Arabidopsis, two RING-finger E3 ubiquitin ligases, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1), influence the photoperiodic flowering pathway (Valverde et al., 2004; Jang et al., 2008; Liu et al., 2008; Lazaro et al., 2012). High CO transcript levels have been detected during the dark phase of both LD and SD conditions, but they do not correlate with CO protein accumulation (Jang et al., 2008). In darkness, COP1 acts as an E3 ubiquitin ligase to ubiquitinate CO, promoting its degradation and consequently repressing flowering (Jang et al., 2008). In the daylight period, CRY-mediated signal may translocate COP1 from the nucleus to the cytoplasm, thereby stabilizing CO, activating FT transcription, and inducing flowering (Liu et al., 2008). In addition, during the daytime, HOS1 is required to degrade CO, and it prevents CO accumulation and FT expression during the early daylight hours (Lazaro et al., 2012). Thus, by directly targeting CO through UPS, tight regulation of CO levels is achieved in response to different daylight regimes in Arabidopsis.

Although several transcriptional factors involved in photoperiodic flowering have been characterized in rice (Tsuji et al., 2011), their direct interaction relationship and posttranscriptional regulation remain largely unknown. To elucidate the mechanism underlying Hd1 accumulation during the photoperiodic flowering in rice, we characterize an Hd1-interacting protein, Heading date Associated Factor 1 (HAF1), which is required for modulation of heading date. HAF1 encodes a C3HC4 RING domain-containing E3 ubiquitin ligase. Moreover, we show that HAF1 physically interacts with Hd1 in vitro and in planta and regulates Hd1 protein abundance during the photoperiod in rice. Mutation of HAF1 significantly delays heading date under both SD and LD conditions. Thus, we propose that HAF1 is required to precisely modulate the timing of Hd1 accumulation and to ensure an appropriate photoperiodic response in rice.

RESULTS

HAF1 Interacts with Hd1 in Yeast Cells

Previous studies showed that Hd1 plays a crucial role in promoting flowering under SD conditions (Yano et al., 2000; Izawa et al., 2002; Hayama et al., 2003). The Hd1 protein contains two functional domains: two tandem repeats of zinc finger domains at the N terminus and the CCT domain at the C terminus (Yano et al., 2000). To elucidate the molecular mechanism of Hd1 mediation of flowering in rice, we performed a yeast two-hybrid assay using Hd1 as bait to identify interacting factors of Hd1. We first investigated the transcriptional activation activity of Hd1 in yeast (Saccharomyces cerevisiae). Autoactivation was detected in the full-length Hd1 and truncated Hd1 fragment constructs (amino acids 78 to 395, 117 to 395, and 326 to 395) (Figure 1A). All fragments contained the CCT domain of Hd1, which indicated that the CCT domain might have a functional transcription activation activity. However, no transcriptional activation of the truncated Hd1 protein (amino acids 1 to 325) was detected (Figure 1A), and this fragment was used as a bait to screen a yeast prey cDNA library prepared from penultimate leaf blades of Zhonghua 11 (ZH11) (O. sativa ssp japonica). Four independent clones containing the complete open reading frame of a gene encoding a finger protein were confirmed to interact with Hd1 (amino acids 1 to 325), but not Hd1 (amino acids 1 to 116 or 1 to 77) (Figure 1B). A BLAST search of the open reading frame against the TIGR Rice Genome Annotation database (http://rice.plantbiology.msu.edu/index.shtml) retrieved a sequence that was annotated as a C3HC4 zinc finger gene (LOC_Os04g55510), which we named Heading date Associated Factor 1 (HAF1).

To investigate whether the C3HC4 zinc finger of HAF1 is required for the interaction with Hd1, a series of truncated HAF1 cDNAs were cloned into the bait constructs, and their transcriptional activation activity was assayed in yeast. All the truncated fragments of HAF1 (amino acids 1 to 112, 99 to 619, 288 to 619, 288 to 667, and 620 to 667) showed no transcriptional activation activity (Supplemental Figure 1). Yeast two-hybrid assay demonstrated that constructs containing the N terminus (amino acids 1 to 112) or C terminus (amino acids 620 to 667) of HAF1 were sufficient for the interaction with Hd1 (Figure 1C). This finding suggests that the C-terminal region of HAF1, which includes the C3HC4 domain, may be involved in the interaction with Hd1. Subsequently, we transformed yeast cells with baits encoding two regions of HAF1 (amino acids 99 to 619 and 288 to 667) and prey vectors with a series of truncated Hd1 cDNA fragments to examine their interaction (Figure 1D). The results indicated that the second B-box zinc finger domain of Hd1 is required for interaction with HAF1, but the CCT domain of Hd1 is dispensable.

HAF1 Physically Interacts with Hd1 in Vivo and In Vitro

To examine whether HAF1 interacts with Hd1 in plant cells, we used bimolecular fluorescence complementation assays to investigate the interaction between the HAF1 and Hd1 in the abaxial
epidermal cells of tobacco (*Nicotiana benthamiana*) leaf (Waadt et al., 2008). Hd1 was fused to the split CFP C terminus (SCC:Hd1), while HAF1 was attached to the split CFP N terminus (HAF1:SCN). Interaction of the two proteins would reconstitute the cyan fluorescent protein, allowing for detection of cyan fluorescence. Leaf epidermal cells were analyzed 5 d after infiltration with *Agrobacterium tumefaciens* harboring the constructs. As anticipated, strong cyan fluorescence was observed in the nuclei of leaf cells when the full-length HAF1 and Hd1 proteins were coexpressed (Figure 2A), suggesting that HAF1 interacts with Hd1 in nuclei. When SCC:Hd1 and HAF1:SCN were expressed in leaf cells separately, no fluorescence was detected (Figures 2B and 2C). These data indicate that the interaction of HAF1 with Hd1 occurs in the nuclei in planta.

Consistent with the yeast two-hybrid and bimolecular fluorescence complementation results, pull-down assays also demonstrated that HAF1 interacts with Hd1 in vitro. Maltose binding protein-tagged HAF1 (MBP-HAF1) and GST-tagged Hd1 (GST-Hd1) fusion proteins were expressed separately. As shown in Figure 2D, MBP-HAF1 bound to GST-Hd1, while MBP-HAF1 did not bind to the negative control (GST). These results suggest that HAF1 physically interacts with Hd1 in vitro.

**Identification of the haf1 Gene in Rice**

To characterize the function of HAF1 in rice, we obtained an haf1 mutant from our rice T-DNA insertional library in variety ZH11 (Wu et al., 2003; Zhang et al., 2007). Analysis of the genomic sequence flanking the T-DNA insertion site in haf1 showed that it is located 1660 bp upstream from the ATG start codon of HAF1 on chromosome 4 (Figure 3A). RT-PCR analysis revealed that HAF1 was suppressed in leaves from plants homozygous for the T-DNA insertion, whereas the expression levels of the other genes surrounding the insertion site were not altered in haf1 compared with the wild type (Supplemental Figure 2A). To investigate the developmental defects of haf1, 23 T1 plants were planted in the field during the 2010 rice-growing season in Wuhan, China, which has natural LD conditions. A pair of gene-specific primers (P1/P2) coupled with a T-DNA-specific primer (P3) were used to test the genotype of the T-DNA insertion site (Figure 3A). Among 23 T1 plants, five plants homozygous for the T-DNA insertion showed a late heading date, while the other plants, either heterozygous or homozygous for the wild type, had a normal heading date (Figure 3B). The phenotype segregation ratio in the T1 family of 23 plants...
The late heading date was caused by a recessive mutation of a single Mendelian locus. All progeny of T2 plants either heterozygous or homozygous for the wild type exhibited normal timing of heading, and all T-DNA insertion homozygotes showed late heading. These results strongly suggested that mutation of the gene \textit{HAF1} results in a late heading date.

To further verify that the late heading date was caused by the mutation of \textit{HAF1}, an 11.51-kb genomic DNA fragment containing the entire \textit{HAF1} coding region and the 2877-bp upstream and 2952-bp downstream sequences was introduced into a \textit{haf1} mutant background (Supplemental Figure 2B). Of 173 plants regenerated, 80 headed at least 2 weeks earlier than the 14 plants transformed with the empty vector (the negative control) (Figure 3C). DNA gel blot hybridization analysis of 38 randomly chosen T0 \textit{HAF1}-transgenic plants revealed that 16 plants contained one copy of the transgene, and the others carried two or more copies (Supplemental Figure 2C). All T1 progenies produced by self-pollination of the single-copy transformants showed segregation of heading date under natural growth conditions. As expected, all transgene-positive segregants restored normal heading date (Supplemental Figure 2D). We therefore concluded that \textit{HAF1} modulates heading date in rice.

Heading Date Characterization of \textit{haf1}

To investigate the heading date of \textit{haf1} under different photoperiodic conditions, we grew \textit{haf1} and corresponding wild type under two artificial daylength treatments: SD conditions (10 h light/14 h dark) and LD conditions (14 h light/10 h dark). Under LD conditions, the heading date of \textit{haf1} (83.40 ± 5.64) was delayed 21 d compared with the wild type (62.54 ± 1.40) (Figures 3D and 3F).

Under the SD conditions, the heading date of \textit{haf1} (70.62 ± 4.33) was delayed 17 d compared with the wild type (53.73 ± 3.61) (Figures 3E and 3F). Thus, the \textit{haf1} mutants showed late heading compared with the wild-type plants under both photoperiodic conditions.

To examine whether reduced growth rate caused late flowering in \textit{haf1}, we next compared the leaf emergence rate of \textit{haf1} and wild-type plants until heading. The leaf emergence rate of the \textit{haf1} mutants was almost indistinguishable from that of the wild-type plants prior to heading under both conditions (Figures 3G and 3H). Heading occurred in the wild-type plants after they produced 12.69 ± 0.75 leaves under LD and 10.92 ± 0.67 leaves under SD (Figures 3G and 3H). The \textit{haf1} plants exhibited a later heading date compared with the wild type and produced more leaves under LD (13.80 ± 0.94 leaves) and SD (12.14 ± 0.53 leaves) (Figures 3G and 3H). These results suggested that the delayed heading dates under both photoperiodic conditions were due to a prolonged floral transition in \textit{haf1}.

With regard to other agronomic traits, such as plant height, tiller number per plant, 1000-grain weight, panicle length, and spikelet number per panicle, \textit{haf1} had no obvious differences compared with the wild type (Supplemental Table 1). These results demonstrated that the mutation in \textit{HAF1} does not have any obvious effects other than causing a later heading date.

Expression Pattern of \textit{HAF1} and Subcellular Localization

To examine the temporal and spatial expression pattern of \textit{HAF1}, we performed RT-qPCR to analyze the transcription levels of \textit{HAF1} under natural LD in selected tissues from the wild type: root and leaf blade at the vegetative stage and the root, leaf blade, leaf sheath, culm, and young panicles at the reproductive stage.
stage. *HAF1* transcripts were detected in all examined tissues, but they were relatively abundant in leaf blade (Supplemental Figure 3A). The preferential expression of *HAF1* in leaf blade is consistent with its involvement in photoperiodic flowering in rice.

Next, we examined the transcript levels of *HAF1* in leaf blades at various developmental stages. The leaf blades were collected at dusk every 5 d from the 16th day after germination under LD conditions. The RT-qPCR results showed that *HAF1* was consistently expressed in leaf blade from the vegetative to the reproductive stage (Supplemental Figure 3B). This expression pattern may indicate that *HAF1* is required for the normal growth of rice.

We also assayed the subcellular localization of the *HAF1* protein. A construct, 35S:*HAF1:*GFP, was made to include the *HAF1* cDNA fused in frame to GFP under the control of cauliflower mosaic virus 35S promoter. Hd1 was fused to CFP as a nuclear protein marker. Rice protoplasts from etiolated seedlings were cotransfected with 35S:*HAF1:*GFP and 35S:Hd1:CFP by polyethylene glycol treatment. The GFP-fused full-length *HAF1* co-localized with CFP-fused Hd1 in the nucleus (Supplemental Figure 3C), suggesting that *HAF1* is a nuclear protein.
Effects of HAF1 on Gene Expression in Photoperiod Pathway

To further investigate the role of HAF1 in photoperiod-controlled flowering in rice, we compared the diurnal expression patterns of main flowering time regulators in the haf1 and wild-type plants under both LD (16 h light/8 h dark) and SD (8 h light/16 h dark) conditions. We monitored transcripts day and night by RT-qPCR at 4-h intervals during a 48-h period. Compared with the examined genes involved in photoperiod flowering, the HAF1 transcript level was very low in the wild-type plant and showed diurnal or circadian patterns under either LD or SD conditions (Figures 4A and 4B). The expression of HAF1 was much more abundant during the dark period than the light period (Figures 4A and 4B). In the haf1 mutants, the HAF1 transcript level was dramatically reduced under both conditions, especially during the dark period (Figures 4A and 4B). HAF1 had no effect on the expression of GI, which showed identical expression patterns in haf1 and the wild type under both conditions (Figures 4C and 4D). However, HAF1 had a considerable effect on the expression of Hd1 and Ehd1. HAF1 mutation suppressed the expression peak of Hd1 during the light period under LD and SD conditions (Figures 4E and 4F). The expression of Ehd1 was almost completely suppressed in haf1 under both conditions (Figures 4G and 4H). Ultimately, the expression of two florigen genes, RFT1 in LD and Hd3a in SD (Tamaki et al., 2007; Komiya et al., 2008), was significantly reduced in haf1 (Figures 4I and 4J). Taken together, these results suggested that HAF1 functions upstream of Hd1, Ehd1, RFT1, and Hd3a in the LD and SD photoperiodic flowering pathway. Thus, HAF1 might be involved in both Hd1-dependent and Ehd1-dependent photoperiodic flowering pathways in rice (Tsuji et al., 2011; Song et al., 2015).

HAF1 Is a RING Finger E3 Ubiquitin Protein Ligase

HAF1 is predicted to encode a 667-amino acid protein containing a C3HC4-type RING finger at the C terminus (Figure 5A). The RING domain in HAF1 contains the conserved consensus sequence C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-C-X2-C-X(4-48)-C-X2-C (Figure 5A), in which X is any amino acid (Borden and Freemont, 1996). E3 ubiquitin ligases are characterized by conserved RING finger domain function (Stone et al., 2005), prompting us to test whether HAF1 also has E3 ubiquitin ligase activity.

We first tested whether HAF1 had enzyme activity for self-ubiquitination. We expressed HAF1 in Escherichia coli fused with MBP and purified MAP-HAF1 from the soluble fraction (Supplemental Figure 4A). Self-ubiquitination of HAF1 was assayed in the presence of E1-activating enzyme, E2-conjugating enzyme, and His-ubiquitin. A polyubiquitination signal was observed by immunoblot using an anti-His antibody (Figure 5B, second lane from the right). The anti-MBP blot analysis also showed that MBP-HAF1 was ubiquitinated (Figure 5B, second lane from the right). As the negative controls, no polyubiquitination of HAF1 was detected when E1, E2, His-ubiquitin, or MBP-HAF1 was absent in the reaction (Figure 5B). This result suggested that HAF1 has E3 ubiquitin ligase activity.

The RING domain has been reported to usually form a stable dimer, which is required for ubiquitin transfer (Liew et al., 2010). A polyclonal antibody was raised against a C-terminal fragment of HAF1 and used to immunoprecipitate MAP-HAF1 from E. coli extracts. The RING domain contained in HAF1 was detected by Western blotting (Figure 5C). The high level of ubiquitination of HAF1 by itself (Figure 5D) suggests that HAF1 likely forms a stable dimer under the reaction conditions used in this study.
calculation for the monomer, we speculated that HAF1 may form a homodimer to be ubiquitinated. The truncated fragments of HAF1 (amino acids 99 to 619, 288 to 667, and 620 to 667) that served as bait were able to interact with full-length HAF1 in the yeast two-hybrid assay (Figure 5C). Homodimerization of HAF1 was further confirmed by pull-down experiment (Figure 5D). Taken together, HAF1, as a RING finger protein, may form a dimer to function as an E3 ubiquitin ligase.

**HAF1 Mediates Ubiquitination of Hd1**

Since HAF1 interacts with Hd1 and HAF1 has E3 ligase activity, we considered the possibility that HAF1 mediates ubiquitination of Hd1. To examine this possibility, we purified the tagged proteins MBP-HAF1 and GST-Hd1 (Supplemental Figure 4B) for ubiquitination assays in vitro, and Hd1 was found to be ubiquitinated by HAF1 in the presence of E1, E2, and His-ub (Figure 6A). With increased amounts of GST-Hd1 protein, the intensity of ubiquitinated GST-Hd1 bands was stronger (as shown in Figure 6A, asterisk from the fifth to eighth lanes from the left), whereas the signals from self-ubiquitination of HAF1 declined (Supplemental Figure 5, top panel). These results indicated that HAF1 mediates ubiquitination of Hd1 in vitro.

To confirm the ubiquitination of Hd1 by HAF1, we employed an efficient in vivo ubiquitination assay by agroinfiltration expression of both substrates and E3 ligases in *N. benthamiana* (Liu et al.,...
We transiently expressed HA-tagged Hd1 (HA-Hd1) via agroinfiltration, HA-Hd1 protein was detected, and Hd1 and GAPDH mRNA expression levels were analyzed by RT-PCR (Figure 6B). When HA-Hd1 and Flag-HAF1 were coexpressed in the same leaf area, HA-Hd1 was rapidly degraded (Figure 6B, first lane from the left), but HA-GFP, an internal control in this experiment, was not (Figure 6B). However, when the 26S proteasome inhibitor MG132 was infiltrated into the same region, HA-Hd1 protein could be detected using the HA antibody (Figure 6B, second lane from the left). Together, these results suggested that HAF1 targets Hd1 for ubiquitin-dependent degradation by the 26S proteasome.

HAF1 Regulates Circadian Accumulation of Hd1

Previous studies speculated that posttranslational regulation of Hd1 protein level might play an important role in determining the heading date of rice (Luan et al., 2009; Ishikawa et al., 2011). Because HAF1 mediates Hd1 degradation in vitro, we examined whether the accumulation of Hd1 depends on HAF1 in vivo. We separately harvested 14-d-old seedling leaves from the wild type and haf1 under SD conditions at Zeitgeber time = 0, when HAF1 is highly expressed (Figure 4B). Total protein extracts were prepared from the leaf tissues and incubated with MBP-Hd1 for cell-free degradation assays. We found that MBP-Hd1 was degraded more rapidly during incubation with protein extracts from the wild type compared with those from haf1 mutants. In the presence of MG132, the rate of MBP-Hd1 degradation was lower when the same protein extracts from the wild type were used (Supplemental Figure 6A). By contrast, no obvious degradation of the control MBP protein and rice ACTIN was observed when using the same plant extracts (Supplemental Figures 5B and 5C), indicating that HAF1 is specifically responsible for Hd1 protein degradation.

Next, we further examined Hd1 rhythmic accumulation in wild-type and haf1 plants entrained in both SD and LD conditions. Consistent with the previous examinations, Hd1 expression was rhythmic in the wild type and haf1 (Figures 4C and 4D), with high expression during the dark periods under both conditions (Figures 4C and 4D). Although expression of Hd1 protein in rice was low (Ishikawa et al., 2011), immunoprotein gel blot analysis using anti-Hd1 antibody showed that Hd1 remained relatively abundant in the light (Figures 6C and 6D). In haf1, Hd1 accumulation showed no clear diurnal rhythm but rather almost constant levels for the time points studied (Figures 6C and 6D). The greater Hd1 accumulation in haf1 than in the wild type suggested that HAF1 controls Hd1 stability in vivo.

Figure 6. HAF1 Ubiquitates Hd1 and Promotes Degradation of Hd1.

(A) HAF1 ubiquitates Hd1 in vitro. GST-Hd1 ubiquitination assays were performed using MBP-HAF1, E1, E2, and His-Ub.

(B) HAF1 and the proteasome regulate Hd1 stability in vivo. Immunoblot analysis of protein extracts corresponding to agroinfiltrated N. benthamiana leaves with the indicated plasmids in the presence or absence of MG132. HA-Hd1 and HA-GFP were detected using anti-HA antibody and FLAG-HAF1 using anti-FLAG antibody. FLAG-HAF1 (HAF1), HA-Hd1 (Hd1), and GAPDH mRNA expression levels were analyzed by RT-PCR.

(C) and (D) Circadian accumulation of Hd1 protein in wild-type and haf1 plants grown under SD and LD conditions. Total protein extracts from 35-d-old plant leaves were loaded into each lane. Hd1 and GAPDH mRNA expression levels were analyzed by competitive RT-PCR. ACTIN abundance was detected as loading control. Anti-Hd1 antibody was used to detect Hd1 abundance. CBB, Coomassie blue staining.
Genetic Interaction of HAF1 and Hd1

To investigate the genetic relationship between HAF1 and Hd1, we generated a homozygous haf1 hd1 double mutant in the same background of ZH11. Previous studies demonstrated that Hd1 promotes flowering under SD conditions, whereas it suppresses flowering under LD conditions (Izawa et al., 2002; Hayama et al., 2003; Ishikawa et al., 2005). To analyze the genetic effect of HAF1 on Hd1, we obtained the hd1 mutant in ZH11 (Luan et al., 2009).

Compared with the wild type, hd1 headed later under SD conditions (Supplemental Figure 7) and earlier under LD conditions (Supplemental Figure 8). Under SD conditions, the haf1 hd1 double mutant headed as late as hd1 plants, indicating that Hd1 is genetically epistatic to HAF1 (Supplemental Figure 8). Taken together, these data indicate that HAF1 may influence heading date mainly through Hd1 under SD conditions. This finding agrees with other studies that show Hd1 plays a crucial role in promoting heading under SD conditions (Yano et al., 2000; Izawa et al., 2002; Hayama et al., 2003). Since the major heading regulators Ehd1 and RFT1 may function independently from Hd1 under LD conditions (Doi et al., 2004; Komiya et al., 2008; Komiya et al., 2009), we propose that HAF1 may participate in heading date regulation by ubiquitination of flowering regulators other than Hd1 under LD conditions.

DISCUSSION

Although ubiquitin-mediated degradation of proteins in the photoperiodic flowering pathway has been well characterized in Arabidopsis, little is known about the molecular mechanism responsible for circadian accumulation of flowering regulators in rice. Here, we identified a C3HC4-type RING-finger E3 ubiquitin ligase, HAF1, as an essential factor for Hd1 abundance in heading date determination of rice. HAF1 interacts with HAF1, and Hd1 is the direct substrate of HAF1 for ubiquitination in vitro and in vivo. Mutation of HAF1 resulted in Hd1 accumulation and delayed flowering, especially under SD conditions. Our results demonstrate that HAF1 has a pivotal role in promoting flowering through regulation of Hd1 accumulation during the photoperiodic response in rice.

HAF1 Is a RING Finger E3 Ubiquitin Ligase

HAF1 encodes a C3HC4-type RING-finger E3 ubiquitin ligase, which contains a zinc finger domain at the C terminus. A BLASTp search of HAF1 against the NCBI reference protein database (http://blast.ncbi.nlm.nih.gov/) retrieved highly similar orthologs in Arabidopsis, indicating that HAF1 might represent an E3 ubiquitin ligase generally present in plants. In Arabidopsis, COP1 is a well-characterized C3HC4-type RING-finger E3 ubiquitin ligase that is also involved in regulating flowering time by directly targeting transcriptional activator CO for degradation in the dark (Jang et al., 2008; Liu et al., 2008). The COP1 protein comprises three recognizable domains: a RING-finger domain, a coiled-coil domain, and seven WD40 repeats (Deng et al., 1992; McNellis et al., 1994), all of which have been implicated in mediating the interaction of COP1 with other proteins (Deng et al., 1992; Wang et al., 2001; Yang et al., 2001; Yu et al., 2008). Although HAF1 contains the conserved C3HC4-type RING-finger domain, none of the other characterized domains were found in HAF1. Our investigation suggested that the C3HC4 RING domain of HAF1 is involved in the interaction with Hd1 (Figure 1C). The RING domain has previously been shown to be required for dimer formation of C3HC4 RING finger E3 ubiquitin ligase (Liew et al., 2010; Feltham et al., 2011; Johnson et al., 2012). C3HC4 RING E3 ligases, such as RAG1 (Rodgers et al., 1998), RNF4 (Plechanovová et al., 2011), and cIAP (Mace et al., 2008), can self-interact to form homodimers. Due to two RING domains possibly being required to spatially accommodate E2 (Plechanovová et al., 2011), homodimerization was linked to the ubiquitination function of RING E3 ligases. Based on yeast two-hybrid analysis and GST pull-down assay, HAF1 was confirmed to physically interact with itself to form a homodimer (Figures 5C and 5D). Homodimerization of HAF1 might be a prerequisite for the ubiquitination of Hd1.

Given that Hd1 and its ortholog CO were shown to be subject to ubiquitin-based degradation mediated by HAF1 and COP1, respectively, HAF1 might exert a corresponding function as COP1 in rice. Previous studies showed that COP1 represses flowering by promoting degradation of CO during the night in Arabidopsis (Jang et al., 2008; Liu et al., 2008). Our investigation revealed that HAF1 promotes flowering through regulation of the ubiquitination of Hd1 in rice. haf1 showed later flowering, but other agronomic traits did not differ compared with the wild type (Supplemental Table 1), suggesting that the sole function of HAF1 may center on the heading date, without any other effects in rice. However, COP1 is a pleiotropic effector that regulates not only the floral transition but also photomorphogenesis in seedlings (McNellis et al., 1996; Schwechheimer and Deng, 2000; Luo et al., 2014). A cop1 mutant displayed constitutive photomorphogenic development in darkness (Deng et al., 1991). A recent study suggested that HAF1 might interact with HAL3 and be involved in a newly discovered pathway in the light-regulated growth of rice (Sun et al., 2009). To investigate whether photomorphogenesis in rice requires HAF1, we compared the length of coleoptiles and the first leaf between haf1 and the wild type under various light conditions, including continuous irradiation with red, far-red, and blue (Bc) for 9 d (Takano et al., 2005). We also performed the same examination in continuous darkness. No apparent differences were found between the wild type and haf1 mutants grown in the dark and various light conditions (Supplemental Table 2). Rice PPS has recently been reported to be an ortholog of Arabidopsis COP1. The pps mutant shows a prolonged juvenile phase and also exhibits photomorphogenesis in the dark. However, COP1, unlike PPS, is not involved in the juvenile-adult phase change (Tanaka et al., 2011). These results suggest that mechanisms different from those in rice have developed to ensure developmental progress.

HAF1 Interacts with Hd1 and Promotes Its Degradation

HAF1 showed obvious diurnal expression patterns with a peak at the beginning of daytime and a trough at dusk (Figures 4A and 4B). The transcripts of Hd1 also displayed diurnal expression patterns, with high expression levels during the night and a peak at midnight (Figures 4E and 4F). The phase of expression pattern in HAF1 is...
opposite to that in *Hd1*, indicating that diurnal expression of *HAF1* might be required for degradation of *Hd1* remnants by the morning. However, how is *HAF1* itself regulated in post-transcriptional level? *COP1* in Arabidopsis is known to be regulated by its suppressor CSU1, which negatively regulates *COP1* abundance by maintaining *COP1* homeostasis in dark-grown seedlings (Xu et al., 2014). The necessary components for *HAF1* degradation need to be investigated in future studies. Additionally, recent studies have suggested that the abundant accumulation of *HAF1* itself and its physical interaction with other proteins may synergistically regulate *HAF1* function toward *Hd1* accumulation in heading date control in rice. Light was shown to play a pivotal role in the *Hd1*-mediated regulation of flowering in rice (Ishikawa et al., 2005, 2011). At dusk, *Hd1* expression increases, and the *Hd1* protein is expected to be present in the dark. Under SD conditions, *Hd1* accumulates at night and activates *Hd3a* expression to promote flowering (Ishikawa et al., 2011). When days become longer, *Hd1* protein is exposed to light at dusk and becomes an inhibitor to suppress flowering (Ishikawa et al., 2011). In *Hd1*-overexpressing plants, high levels of *Hd1* may exist in the light at dusk, resulting in delayed flowering under SD conditions (Ishikawa et al., 2011). Previous studies suggested that *Hd1* mediates regulation of *Hd3a* expression mainly through PhyB (Ishikawa et al., 2005, 2011). Since *Hd1* is the direct target of *HAF1* for ubiquitination, the increased levels of *Hd1* protein appear in the *haf1* mutant during the daytime (Figure 6C). In the presence of afternoon light, modified *Hd1* acts as a repressor of *Hd3a* expression, resulting in delayed flowering of *haf1* under SD conditions.

**HAF1 May Participate in Heading Date Control under LD Conditions**

Our investigation suggested that *HAF1* influences the *Hd1* and *Ehd1*-dependent pathways (Figure 4), which induce flowering of rice under SD and LD conditions, respectively. In *haf1* mutants, the transcript levels of *Hd3a* and *RFT1* were decreased (Figures 4I and 4J), suggesting that *HAF1* is involved in the photoperiodic regulation of flowering in rice. Under both photoperiodic conditions, the expression of *Ehd1* was almost eliminated in *haf1* mutants (Figures 4G and 4H), suggesting that *HAF1* is also required for *Ehd1* expression under LD conditions. *Ehd1* has been demonstrated to be a unique activator to induce flowering in rice independently of *Hd1* (Doi et al., 2004). Considering that the transcript level of *Ehd1* is downregulated in plants overexpressing *Hd1* (Ishikawa et al., 2011), the accumulation of *Hd1* protein in *haf1* might repress the expression of *Ehd1* under SD and LD conditions. Furthermore, under LDs, the *haf1* *hd1* double mutant flowers as late as *haf1* (Supplemental Figure 8), indicating that *HAF1* may have a crucial role in the photoperiod pathway under LD conditions. Recently, a photoperiodic flowering pathway, ELF3-Ghd7-*Ehd1*-RFT1 (Tsujii et al., 2011; Zhao et al., 2012; Yang et al., 2013), was proposed for the induction of rice flowering under LD conditions. We speculate that additional components may interact with *HAF1* and be regulated by *HAF1* at the posttranscriptional level to mediate flowering progress in LD conditions.

**METHODS**

**Plant Materials and Growth Conditions**

T-DNA-tagged mutant *haf1* was identified from our T-DNA insertional mutant library (http://rmd.ncgr.cn/; Wu et al., 2003). Screening of heading date mutants was performed by planting the mutant materials under natural LD in the experimental field of Huazhong Agriculture University at Wuhan (30.4°N, 114.2°E), China, in the summer of 2010. We also planted the *haf1* and wild-type plants under artificial SD (10 h light/14 h dark, 28°C) and LD (14 h light/10 h dark, 28°C) conditions in a growth control room (BB-Eshengtaihe Ctrl Tech). Heading date was recorded as the number of days from germination to the day of panicle emergence from the leaf sheath.

We grew the seedlings at 28°C in a Four Color LED Absolute Cold Light plant incubator (Biotron LH-55LED-SS; Nippon NK System) under different monochromatic light sources, far red (735 nm), red (670 nm), and blue (470 nm), to examine the role of *HAF1* in photomorphogenesis.

**Genotyping of Mutant Plants**

Genotyping of the *haf1*-segregating population was performed by PCR using the following primers: P1, P2, and P3. PCR was conducted with an initial step of 94°C incubation for 5 min; a second step of 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min and final products of 463 and 1289 bp. Genotyping of the *hdf1* was performed as described by Luan et al. (2009). Leaf emergence rate was calculated according to the measurements described by Itoh et al. (1998).

**Yeast Two-Hybrid Assay**

Total RNA for construction of the yeast AD-fused cDNA library was prepared from penultimate leaf blades of *ZH11* using Matchmaker Library Construction and Screening Kits (Clontech). The library was screened using truncated *Hd1* protein (amino acids 1 to 325) as bait by yeast mating. Plasmids from positive clones were verified for interaction by transformation into yeast (*Saccharomyces cerevisiae*) AH109 cells containing pGBK7-Hd1 (amino acids 1 to 325), pGBK-LAX2(156-394)/pGAD-LAX1 was the positive control (Tabuchi et al., 2011).

To test *Hd1* and *HAF1* transcriptional activity, the full-length or truncated CDS of *Hd1* and *HAF1* were amplified and cloned into the pGBK7 (Clontech), respectively. The assays for transactivation activity were then performed following the manual of Matchmaker Gold Yeast Two-Hybrid System (Clontech). Transformants were plated on either tryptophan-negative synthetic dropout medium (SD/Trp- ) or tryptophan-negative and adenine-negative synthetic dropout medium (SD/Trp-/Ade- ).

**Bimolecular Fluorescence Complementation Assays**

The cDNAs of *Hd1* and *HAF1* were individually cloned into pSCYCE-R and pSCYNE vectors that contained either N- or C-terminal GFP fragments (Waadt et al., 2008), designated SCC-Hd1 and HAF1:SCN, respectively. The bimolecular fluorescence complementation assays using *Nicotiana benthamiana* (tobacco) were performed as described by Yuan et al. (2012).

**Vector Construction and Rice Transformation**

An 11.51-kb *BgIII*-BgIII genomic DNA fragment containing the entire *HAF1* coding region and the 2877-bp upstream and 2952-bp downstream sequences was isolated by digestion of the Clemson BAC clone OSUN-Ba0010D21 (kindly provided by R. Wing, University of Arizona) and inserted into the binary vector pCAMBIA2301 (Cambia). An empty pCAMBIA2301 vector was used as a control. Constructs were introduced into Agrobacterium tumefaciens EHA105 and transformed into homozygous calli from *haf1* by Agrobacterium-mediated transformation as previously
Fresh leaves were harvested from DNA Extraction and DNA Gel Blot Hybridization (Leica TCS SP2) after incubation at 28°C for 12 to 16 h.

**Subcellular Localization of HAF1**

To construct the subcellular localization plasmids, the full-length CDS of HAF1 was cloned into a pM999-GFP vector that contained GFP reporter gene (primers are described in Supplemental Data Set 1). Protoplasts were isolated from 10- to 15-d-old rice (Oryza sativa) etiolated seedlings and transformed with the pM999-CFP-Hd1. Fluorescence in the transformed protoplasts was captured using a confocal laser scanning microscope (Leica TCS SP2) after incubation at 28°C for 12 to 16 h.

**DNA Extraction and DNA Gel Blot Hybridization**

Fresh leaves were harvested from field-grown plants, and genomic DNA was extracted using the CTAB method described by Murray and Thompson (1980). The probes for DNA gel blot hybridization were the PCR products amplified with T-primers (primers are described in Supplemental Data Set 1). The experimental procedures for DNA gel blot analysis followed methods previously described by Liu et al. (1997).

**Gene Diurnal Expression Analysis**

Rice seedlings were grown for 30 d in the greenhouse under natural daylength conditions. For LD samples, the plants were transferred to a growth chamber set for SD (8 h light/16 h dark, 28°C and 50% humidity) and entrained for 5 d. Then, penultimate leaves were harvested every 4 h during a 48-h period from wild-type and haf1 plants. For SD samples, plants were transferred to a growth chamber set for SD (8 h light/16 h dark, 28°C and 50% humidity) and entrained for 5 d. Samples were collected in the same way for the LD conditions.

**Total RNA was extracted from various tissues using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions, and first-strand cDNA was synthesized from 2.5 μg total RNA with SuperScript III reverse transcriptase (Invitrogen). RT-qPCR was performed with the Applied Biosystems 7500 real-time PCR detection system using SYBR Green Master Mix (Applied Biosystems). The measurements were obtained using the relative quantification method (Livak and Schmittgen, 2001).**

**Purification of Recombinant Proteins**

Full-length Hd1 cDNA was inserted into a GST fusion vector pGEX-4T-1 (Pharmacia). The resulting GST-Hd1 fusion was expressed in BL21-CodonPlus (Stratagene) Escherichia coli cells and purified using glutathione Sepharose 4B (Pharmacia). MBP-tagged HAF1 protein and MBP alone were expressed in BL21-CodonPlus and purified using amylose resin beads (New England Biolabs).

**Pull-Down Assays**

The coding sequences of HAF1 and Hd1 were cloned into the pGEX-4T-1 vector to generate the constructs to express GST-HAF1 and GST-Hd1, respectively. The method for in vitro pull-down assays was modified from Xia et al. (2013). Bacterial lysates containing ~15 mg GST-Hd1 or GST-HAF1 fusion proteins were mixed with lysates containing ~30 mg MBP-HAF1 fusion proteins. Glutathione Sepharose (30 μL; GE Life Sciences) was added with rocking at 4°C for 60 min. Beads were washed four times with the TGH buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and 1× Complete protease inhibitor cocktail [Roche]), and the isolated proteins were further separated on two 10% SDS-PAGE gels and detected by immunoblot analysis with anti-GST antibody (Abmart M20007L; 1:1000 dilution) and anti-MBP antibody (NEB E0032S; 1:10,000 dilution), respectively.

**Ubiquitination Assays in Vitro**

Reaction mixtures (30 μL) contained 110 ng E1 (Boston Biochem), 170 ng E2 (UBChn3a; Boston Biochem), 1 mg His-ubiquitin (Boston Biochem), and 2 mg MBP-HAF1 fusion protein and various concentrations of GST-Hd1 fusion protein in reaction buffer containing 50 mM Tris, pH 7.5, 3 mM DTT, 5 mM MgCl₂, and 2 mM ATP. After 6 h incubation at 30°C, reactions were stopped by adding sample buffer, and half of the mixtures were separated onto two 10% SDS-PAGE gels. Ubiquinated GST-Hd1 was detected using anti-GST antibody (Abmart M20007L; 1:1000 dilution) and anti-HIS antibody (Abmart M20001L; 1:1000 dilution). Images were visualized on Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology).

**Ubiquitination Assay in Vivo**

In vivo ubiquitination assay was performed according to the protocol described by Liu et al. (2010). We coinfiltated the Agrobacterium strains carrying the Flag-HAF1 and HA-Hd1 plasmids into N. benthamiana leaves. The corresponding empty vectors were used as the controls, and the HA-GFP plasmid was added as an internal control. For proteasome inhibition, leaves were infiltrated with 10 mM MG132 (Sigma-Aldrich) solution for 12 h before sample collection. Three days after infiltration, samples were collected for protein and RNA extraction.

**Hd1 Polyclonal Antibody Preparation**

For preparation of Hd1 polyclonal antibody, a 960-bp DNA fragment encoding a 320-amino acid peptide of Hd1 (residues 1 to 320) was cloned into pET32a vector (Novagen). The recombinant protein was expressed in E. coli DE3 (Transgen) and purified using nickel nitrioltriacetic acid agarose (Qiagen) to produce rabbit polyclonal antibodies (prepared by Abcolonal of China). The antibody (1:200 dilution) was tested by immunoblot analysis using purified GST-Hd1 and MBP-Hd1 from *E. coli*.

**Detection of Hd1 Abundance in Rice**

Rice leaves from 35-d-old seedlings were ground into a fine powder in liquid nitrogen. A 200-μL aliquot of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, and 1× Complete protease inhibitor cocktail [Roche]) was added to each 100-mg powder sample. The mixture was vortexed and then chilled on ice for 5 min. Samples were centrifuged at 16,000g for 20 min at 4°C, and the supernatant was collected and stored at ~70°C. An equal amount of rice protein was loaded and separated by SDS-PAGE and then stained by Coomassie Brilliant Blue or detected by immunoblot analysis with anti-ACTIN antibody (Abmart M20009L; 1:1000 dilution). For detection of Hd1 abundance, an equal amount of rice protein was separated on a 10% SDS-PAGE and detected by immunoblot analysis with anti-Hd1 antibody (1:200 dilution).

**Cell-Free Protein Degradation Assay**

For cell-free protein degradation assays, seedlings were grown in greenhouse for 10 to 14 d under SD conditions. Total proteins were extracted in the degradation buffer (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 5 mM DTT, and 10 mM ATP). To monitor the degradation of the expressed recombinant MBP-Hd1 and MBP proteins, 200 ng purified MBP-Hd1 and MBP protein was added to 75 μL seedling extract for individual assays. The reaction mixtures were incubated at 30°C for the indicated time points. Reactions were blocked by adding the sample buffer. An equal amount of...
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AUTHOR CONTRIBUTIONS

C.W., Y.Y., and D.F. conceived this project and designed the research. Y.Y. identified the haf1 mutant and performed the genetic transformation. Y.Y., D.F., C.Z., Y.Z., H.Z., and T.L. performed the genetic analysis. D.F., C.Z., and X.L. performed the ubiquitin activities analyses. C.Y. supervised the study. C.W., Y.Y., and D.F. analyzed the data and wrote the article.

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The RING-Finger Ubiquitin Ligase HAF1 Mediates Heading date 1 Degradation during Photoperiodic Flowering in Rice
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