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Ef7 Encodes an ELF3-like Protein and Promotes Rice Flowering by Negatively Regulating the Floral Repressor Gene *Ghd7* under Both Short- and Long-Day Conditions

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Much progress has been made in our understanding of photoperiodic flowering of rice and the mechanisms underlying short-day (SD) promotion and long-day (LD) repression of floral induction. In this study, we identified and characterized the *Ef7* gene, one of the rice orthologs of Arabidopsis *EARLY FLOWERING 3* (*ELF3*). The *ef7* mutant HS276, which was induced by γ-irradiation of the *japonica* rice cultivar 'Gimbozu', flowers late under both SD and LD conditions. Expression analyses of flowering time-related genes demonstrated that *Ef7* negatively regulates the expression of *Ghd7*, which is a repressor of the photoperiodic control of rice flowering, and consequently up-regulates the expression of the downstream *Ehd1* and *FT*-like genes under both SD and LD conditions. Genetic analyses with a non-functional *Ghd7* allele provided further evidence that the delayed flowering of *ef7* is mediated through the *Ghd7* pathway. The analysis of light-induced expression of *Ghd7* revealed that the *ef7* mutant was more sensitive to red light than the wild-type plant, but the gate of *Ghd7* expression was unchanged. Thus, our results show that *Ef7* functions as a floral promoter by repressing *Ghd7* expression under both SD and LD conditions.

Keywords: *EARLY FLOWERING 3* • Late-flowering mutant • Photoperiodic flowering • Rice (*Oryza sativa* L.).

Abbreviations: BAC, bacterial artificial chromosome; CO, CONSTANS; DD, constant darkness; ELF3, EARLY FLOWERING 3; FT, FLOWERING LOCUS T; GI, GIGANTEA; GUS, β-glucuronidase; LL, constant light; LD, long day; ND, natural daylength; NLS, nuclear localization signal; PHYB, phytochrome B; RT–PCR, reverse transcription–PCR; SD, short day; WT, wild type.

The nucleotide sequences reported in this paper have been submitted to the DNA Data Bank of Japan under accession numbers AB686539 (Gimbozu allele) and AB686540 (HS276 allele).

Introduction

In plants, the timing of floral transition has a direct impact on reproductive success. The floral transition is triggered by both endogenous and environmental factors. One of the most important environmental factors for floral transition is the change in daylength (photoperiod). While other environmental factors vary from year to year, daylength change follows a predictable pattern. Therefore, a large majority of plants, except those that originated in low latitudes, have evolved mechanisms to integrate the response to daylength changes into the pathways regulating floral initiation.

Genetic and molecular analyses in the model plant *Arabidopsis thaliana*, a long-day (LD) plant, have led to the identification of a number of genes responsible for the photoperiodic regulation of flowering. These studies have demonstrated that the photoperiodic response is regulated by the involvement of light perception and the circadian clock (Searle and Coupland 2004, Imaizumi and Kay 2006). *EARLY FLOWERING 3* (*ELF3*), one of the genes controlling photoperiod response, encodes a highly conserved nuclear protein in plants (Hicks et al. 1996, McWatters et al. 2000, Covington et al. 2001, Hicks et al. 2001, Liu et al. 2001). The ELF3 protein has a number of binding partners, including the red light photoreceptor phytochrome B (PHYB) and clock-related GIGANTEA (GI) proteins, to integrate the light input into the circadian

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clock into regulation of photoperiodic flowering (Reed et al. 2000, Kim et al. 2005, Yu et al. 2008, Yoshida et al. 2009, Dixon et al. 2011, Nefissi et al. 2011, Kolmos et al. 2011). ELF3 mediates the circadian gating of light responses by associating with PHYB, thus regulating light input into the clock.

Rice (*Oryza sativa* L.) is classified as a facultative short-day (SD) plant: flowering is accelerated under SD conditions and delayed under LD conditions (Thomas and Vince-Prue 1997). Under SD conditions, flowering is promoted by the transcription of *Heading date 3a* (*Hd3a*) and *Rice FT-like 1* (*RFT1*) [rice orthologs of Arabidopsis *FLOWERING LOCUS T* (*FT*)], which are activated independently by *Heading date 1* (*Hd1*) and *Early heading date 1* (*Ehd1*) (Yano et al. 2000, Izawa et al. 2002, Kojima et al. 2002, Doi et al. 2004, Tamaki et al. 2007, Komiya et al. 2008, Komiya et al. 2009, Takahashi et al. 2009, Tsuji et al. 2011). *Hd1* encodes a rice ortholog of Arabidopsis *CONSTANS* (*CO*), and *Ehd1* encodes a B-type response regulator without any clear orthologs in Arabidopsis (Yano et al. 2000, Doi et al. 2004). Under LD conditions, flowering is delayed by *Hd1* repression of downstream *FT*-like genes and by the down-regulation of *Ehd1* expression (Yano et al. 2000, Kojima et al. 2002, Ishikawa et al. 2011). *Grain number, plant height and heading date 7* (*Ghd7*), which encodes a CCT (CO, CO-LIKE and TIMING OF CAB1) motif-containing protein, functions as a flowering repressor together with *Hd1* under LD conditions (Xue et al. 2008, Itoh et al. 2010). *Ghd7* expression is regulated by light- and circadian clock-dependent gating (Itoh et al. 2010). *Ghd7* is acutely induced when phytochrome signals coincide with a photosensitive phase, and Ghd7 represses *Ehd1* expression (Itoh et al. 2010). Recently, it was reported that the gene *Days to heading 8* (*DTH8*)/*Grain number, plant height and heading date 8* (*Ghd8*)/*Heading date 5* (*Hd5*) delayed flowering under LD conditions but promoted it under SD conditions, and that DTH8/Ghd8/Hd5 might form a complex with Hd1 to control flowering (Wei et al. 2010, Yan et al. 2011).

Much progress has been made in our understanding of the photoperiodic regulation of floral induction in rice, but the molecular regulation of photoperiodic flowering remains unclear. We previously identified a novel flowering time locus, referred to as *early flowering 7* (*ef7*), from mutant line HS276, which showed late flowering under SD, LD and natural daylength (ND) conditions (Yuan et al. 2009). *ef7* is a single recessive allele located within a 129.5 kb genomic region on chromosome 6 (Yuan et al. 2009). In this study, we identified the *Ef7* gene by sequencing and complementation analysis. Here, we show that *Ef7* is a rice ortholog of Arabidopsis *ELF3* that regulates flowering time via the *Ghd7* pathway under both SD and LD conditions. In the *ef7* mutant, the expression of *Ghd7* was up-regulated under both SD and LD conditions, consequently down-regulating the expression of the downstream genes (*Ehd1* and *FT*-like genes) under both conditions. Thus, *Ef7* functions as a negative regulator of *Ghd7*, promoting flowering under both SD and LD conditions.

Results

Phenotypes of the *ef7* mutant

The *ef7* mutant, line HS276, is a late-flowering mutant induced by γ-irradiation of seeds of the *japonica* rice cultivar 'Gimbozu'. It flowered 3.8 d later (102.5 ± 0.4 d) than the wild type (WT) (98.7 ± 0.6 d) under ND conditions (**Supplementary Fig. S1A**). To investigate whether the mutation affects the photoperiod response, we grew the *ef7* mutant and the WT under various daylength conditions. Under SD conditions of 10, 12 and 13 h light, the *ef7* mutant flowered 6.3, 8.7 and 10.0 d, respectively, later than the WT, while under LD conditions of 14 and 14.5 h light, it flowered 19.9 and 40.9 d, respectively, later than the WT (**Fig. 1A**). The difference in flowering time between the *ef7* mutant and the WT under LD conditions was larger than that under SD conditions. These results reveal that *Ef7* functions as a floral promoter under both SD and LD conditions.

We compared several morphological traits—culm length, number of panicles per plant, panicle length, number of grains per panicle, percentage of ripened grains and 1,000 grain weight—of the *ef7* mutant with those of the WT plants under ND conditions. The number of panicles per plant was higher and panicle length was significantly shorter in the WT than in the *ef7* mutant (**Fig. 1B**). On the other hand, no significant differences were found in culm length, percentage of ripened grains, number of grains per panicle or 1,000 grain weight (**Fig. 1B**). In addition, the grain of the *ef7* mutant had a long awn while the WT grain had a very short awn (**Supplementary Fig. S1B**). No other differences such as in leaf color or leaf shape were observed (**Fig. 1C**).

Cloning of the *ef7* mutant gene

We previously reported that the *Ef7* locus was delimited within a 129.5 kb region on the short arm of chromosome 6 (Yuan et al. 2009). A database search [the Rice Annotation Project Database (RAP-DB); http://rapdb.dna.affrc.go.jp, Tanaka et al. 2008] detected 15 putative protein-coding sequences in this region (**Fig. 2A**). First, we selected a bacterial artificial chromosome (BAC) clone that included the candidate region of *ef7* and analyzed its nucleotide sequence. We compared the sequence with that of the *japonica* cultivar 'Nipponbare' in RAP-DB and found a total of 11 sequence polymorphisms in four genes (**Supplementary Table S1**). When the sequences of these four genes were compared between the *ef7* mutant and the WT (Gimbozu), the only differences were found in Os06g0142600, in which the *ef7* mutant harbored two 8 bp deletions and three 1 bp substitutions relative to the WT (**Supplementary Table S1**, **Fig. 2B**). To determine the full-length cDNA sequence of Os06g0142600, we sequenced a product amplified by reverse transcription–PCR (RT–PCR) with 3′- and 5′-primer extension. Os06g0142600 consisted of four exons and three introns, and the polymorphisms between the *ef7* mutant and the WT were located in exon 4 (**Fig. 2B**).

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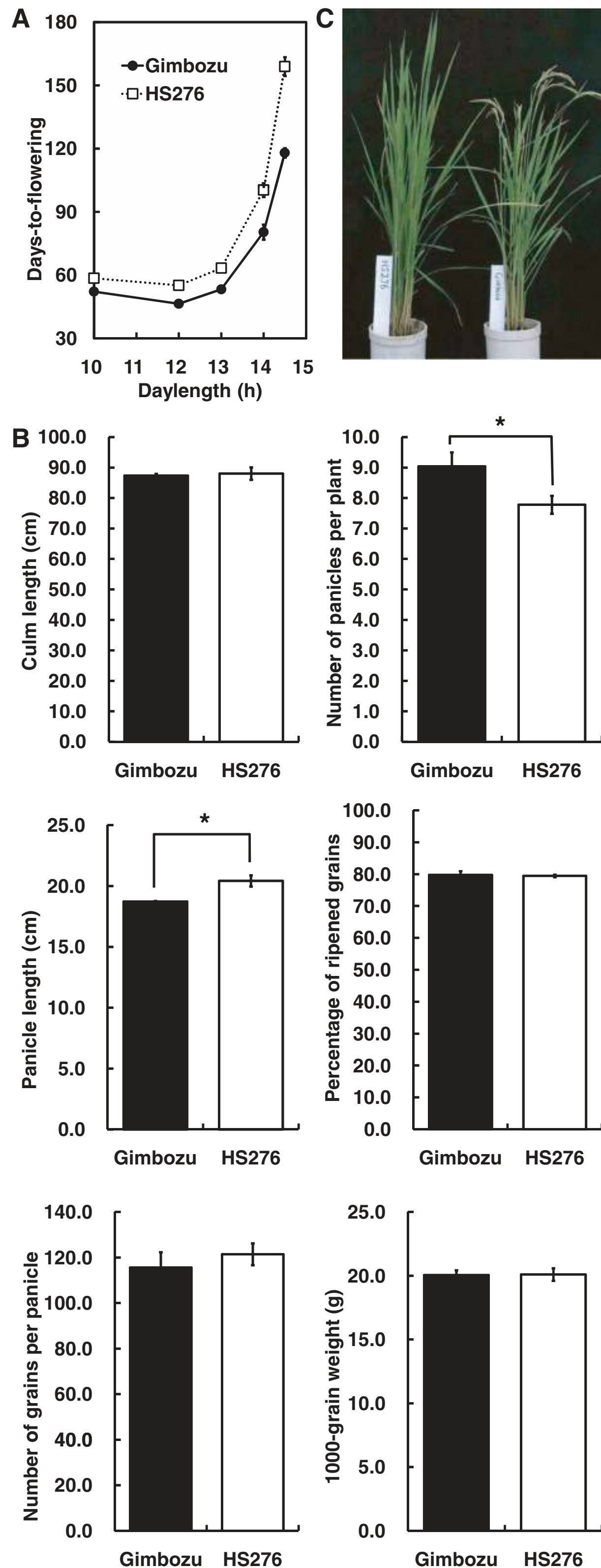

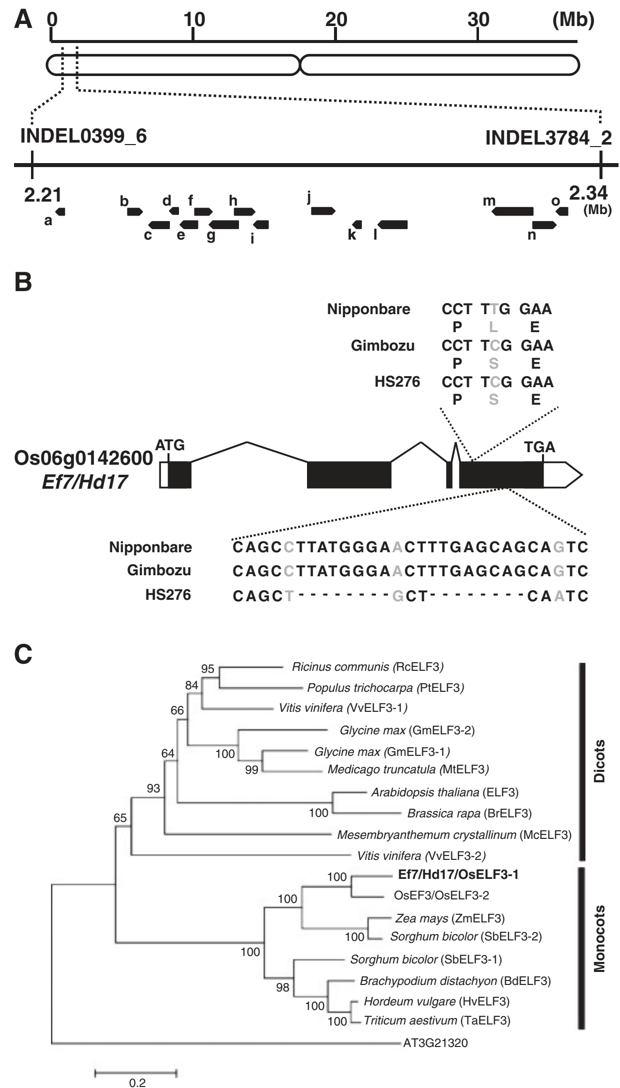

Fig. 2 Identification of the *Ef7* gene and phylogenetic analysis of the Ef7 protein. (A) Location of the *Ef7* candidate region on chromosome 6. Box arrows indicate putative open reading frames annotated in the Rice Annotation Project Database (http://rapdb.dna.affrc.or.jp). (B) Schematic diagram of the *Ef7* gene and polymorphism among Nipponbare, the WT (Gimbozu) and the *ef7* mutant (HS276). White and black boxes indicate untranslated regions (UTRs) and exons, respectively. Lines connecting black boxes indicate introns. (C) Phylogenic relationship of ELF3-like proteins from various plant species. Gene IDs and locus number are shown in **Supplementary Fig. S2**.

Fig. 1 Phenotypes of the *ef7* mutant (HS276) and the WT (Gimbozu). (A) Days to flowering under different photoperiodic conditions. Values are means ($n = 10$). Error bars indicate standard deviations. (B) Comparison of culm length, number of panicles per plant, panicle length, percentage of ripened grains, number of grains per panicle and 1,000 grain weight under natural day (ND) conditions. Values are means ($n = 3$, with 10 plants per replicate). Error bars indicate standard errors. Asterisks indicate a significant difference at the 5% level by a two-tailed Student's *t*-test. (C) WT (right) and *ef7* mutant (left) under ND conditions.

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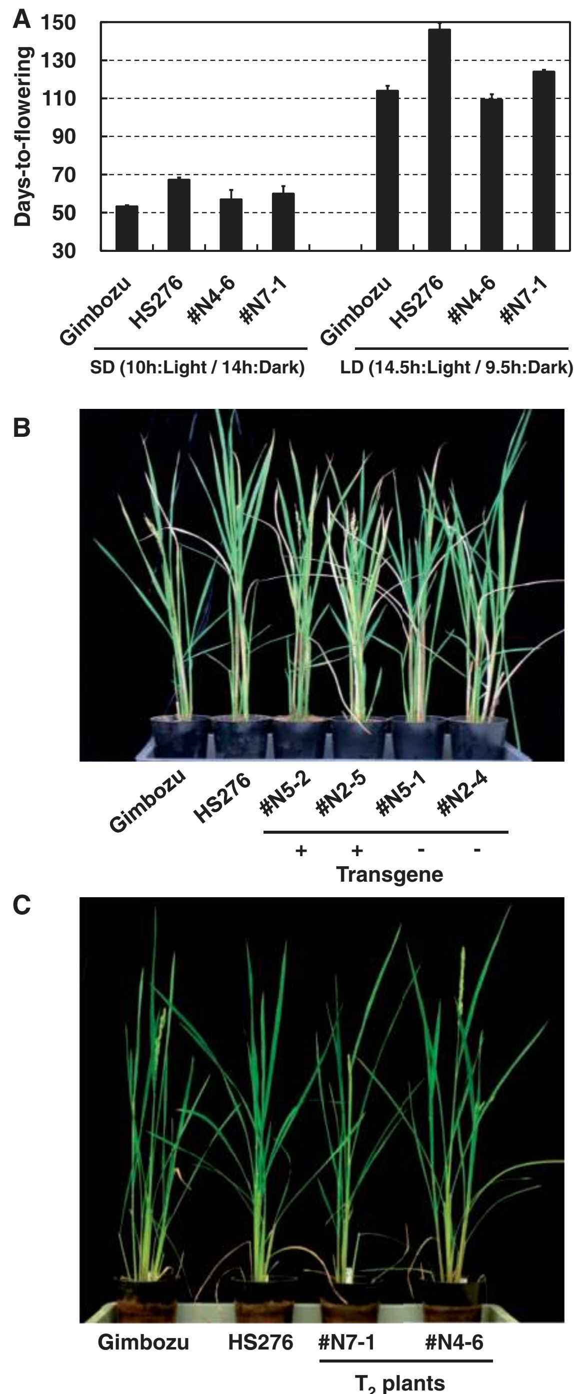

Fig. 3 Comparison of phenotypes of the WT (Gimbozu), the *ef7* mutant (HS276) and *ef7* complemented with the *Ef7* coding sequence. (A) Days to flowering under short-day (SD) and long-day (LD) conditions. Values are means ($n = 8$). Error bars indicate standard deviations. (B) WT (Gimbozu), *ef7* mutant (HS276) and T_1 segregants under LD conditions. The presence of the transgene was determined by TaqMan quantitative RT–PCR (qRT–PCR) described in the Materials and Methods. (C) WT (Gimbozu), *ef7* mutant (HS276) and transgene-containing T_2 plants under SD conditions.

Os06g0142600 (*OsELF3-1*) is predicted to encode a 760 amino acid protein and is an ortholog of the Arabidopsis flowering time gene *ELF3*; the predicted OsELF3-1 protein sequence is 38.3% identical to that of Arabidopsis ELF3 (**Supplementary Fig. S2**). ELF3 homologs were found in both dicots and monocots, and five motifs were highly conserved among plants (**Fig. 2C**; **Supplementary Fig. S2**). To demonstrate that the *ef7* mutant phenotype was caused by the lesions in the ELF3-like protein, we complemented *ef7* by introducing a 9.4 kb genomic segment of the WT, which included a 2.9 kb region upstream from the transcriptional start site, the putative coding region of *OsELF3-1* and a 2.1 kb downstream sequence. T_1 and T_2 plants containing this segment clearly showed earlier flowering than the *ef7* mutant (**Fig. 3**). These results confirm that *Ef7* encodes an ELF3-like protein.

Expression pattern of the *Ef7* gene

We investigated the diurnal expression of the *Ef7* gene in the WT and the *ef7* mutant. In the WT, *Ef7* expression started to increase after midnight, peaked just at dawn and gradually decreased during the daytime under SD conditions. In the *ef7* mutant, the expression followed a similar pattern but was significantly lower at most time points (**Fig. 4A**). We carried out *Ef7* promoter::GUS (β-glucuronidase) reporter analysis to profile the *Ef7* expression. *GUS* expression was detected in mesophyll cells of the young leaves, anthers, stigmas and the top of the lemmas (**Fig. 4B–F**).

Expression of photoperiodic flowering pathway genes in the *ef7* mutant under SD and LD conditions

To identify potential downstream genes regulated by *Ef7*, we examined the expression levels of five flowering-related genes (*Hd1*, *Ghd7*, *Ehd1*, *Hd3a* and *RFT1*). Leaf samples were collected from 30- and 80-day-old plants grown under SD and LD conditions, respectively.

Under SD conditions, the peaks of *Ehd1* and *Hd3a* expression were lower in the *ef7* mutant than in the WT, but *RFT1* expression did not show any obvious difference between the two genotypes (**Fig. 5**). On the other hand, the peaks of *Hd1* and *Ghd7* expression were higher in the *ef7* mutant than in the WT (**Fig. 5**). Under LD conditions, the peaks of *Ehd1*, *Hd3a* and *RFT1* expression were lower in the *ef7* mutant than in the WT, while those of *Hd1* and *Ghd7* were higher in the *ef7* mutant than in the WT (**Fig. 5**). *Ghd7* acts as a repressor of *Ehd1* expression, and thereby delays flowering under LD conditions (Xue et al. 2008, Itoh et al. 2010). Our results therefore suggest that *Ef7* negatively regulates the expression of *Ghd7* and *Hd1*, and consequently up-regulates the expression of the genes downstream of *Ghd7* (i.e. *Ehd1* and *FT*-like genes) under both SD and LD conditions.

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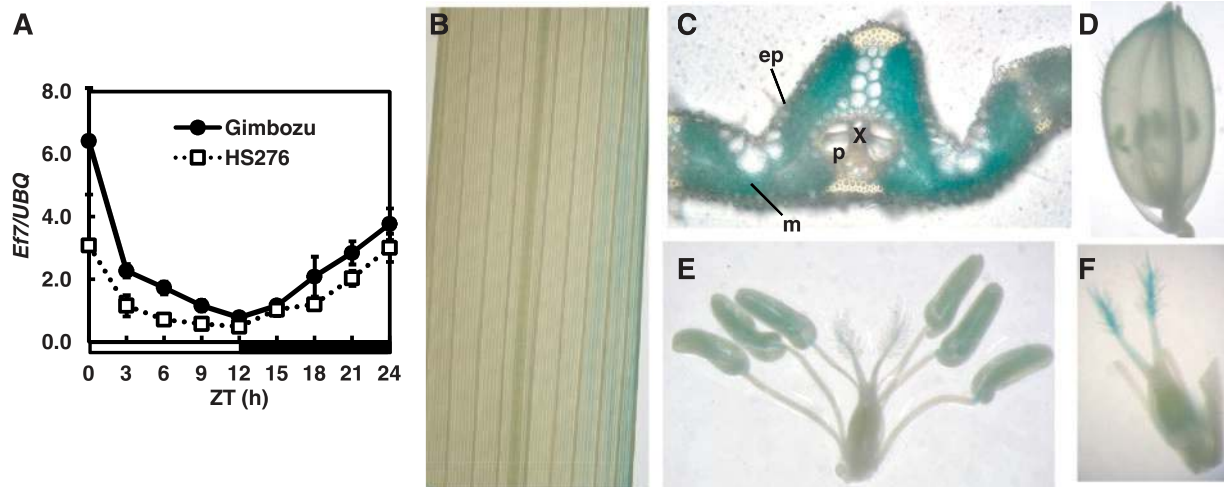

Fig. 4 (A) Comparison of the expression level of *Ef7* between the WT (Gimbozu) and the *ef7* mutant (HS276) under SD conditions. Error bars indicate standard deviations ($n = 3$). ZT, Zeitgeber time (h). (B–F) Localization of *GUS* expressions in different tissues. Transgenic rice plants containing an *Ef7* promoter::*GUS* fusion were stained for the detection of GUS activity. (B) Leaf. (C) Cross-sectioned leaf. (D) Lemma and palea. (E) Stamen and pistil. (F) Magnified view of a pistil. ep, epidermis; m, mesophyll; p, phloem; x, xylem.

Effect of *Ef7* on flowering time mediated through the *Hd1* and *Ghd7* pathways

To confirm whether the delayed flowering phenotype of the *ef7* mutant is mediated through the *Ghd7* and *Hd1* pathways under both SD and LD conditions, we examined the effect of *Ef7* on flowering time when a defective allele of either *Ghd7* or *Hd1* loci was present in the genome.

Under SD conditions, the *hd1* mutant flowered later than the WT, while the *ghd7* mutant flowered earlier than the WT. The *hd1*/*ef7* double mutant flowered later than the *hd1* mutant and at the same time as the *ef7* mutant. The *ghd7*/*ef7* double mutant flowered at about the same time as the WT, which was slightly later than the *ghd7* mutant and earlier than the *ef7* mutant (**Fig. 6**). Under LD conditions, both the *hd1* and *ghd7* single mutants flowered much earlier than the WT. The *hd1*/*ef7* double mutant flowered slightly later than the *hd1* mutant and much earlier than the *ef7* mutant, while the *ghd7*/*ef7* double mutant flowered at the same time as the *ghd7* mutant and much earlier than the *ef7* mutant (**Fig. 6**). These results suggest that *Hd1* and *Ghd7* genetically interact with *Ef7* under both SD and LD conditions. Under SD conditions, *Ef7* is required to promote flowering through the *Hd1* pathway, and the delayed flowering of *ef7* is mediated through the *Ghd7* pathway. Under LD conditions, *Ef7* functions via the *Hd1* and *Ghd7* pathways to regulate flowering, and these pathways might interact with each other.

Effect of *Ef7* on light-induced expression of *Ghd7*

The response of *Ghd7* to red light is modulated by circadian rhythm, a mechanism referred to as gated phytochrome signaling (Itoh et al. 2010). We thus examined the gated expression pattern of *Ghd7*. Red light pulses induced *Ghd7* expression in the WT: this expression was gated with a red light-inducible phase pattern clearly peaking at subjective dawn (**Fig. 7**). The gated expression pattern of the *ef7* mutant did not differ from that of the WT, but the levels of expression were significantly higher in the *ef7* mutant (**Fig. 7**). This high expression of *Ghd7* in the *ef7* mutant was consistent with the results of the diurnal expression analyses (**Fig. 5**). These results indicate that *Ef7* also functions as a repressor of light-induced expression of *Ghd7*.

Expression of clock-regulated genes in the *ef7* mutant

Arabidopsis ELF3 has a pivotal function to integrate the light signal into the circadian clock (Hicks et al. 1996, McWatters et al. 2000, Covingtom et al. 2001). To examine circadian clock-regulated gene expression in the *ef7* mutant, we analyzed the expression of a luciferase (*LUC*) gene driven by the *CHLOROPHYLL a/b-BINDING PROTEIN* promoter (*Cab1R:LUC*) under a light/dark cycle, constant light (LL) and constant dark (DD) conditions. *Cab1R:LUC* expression was quickly induced by light at dawn under a light/dark cycle (**Fig. 8A**). The peak of induction in the *ef7* mutant seedlings occurred 1–4 h earlier than in the WT. *Cab1R:LUC* expression under DD conditions was not affected in the *ef7* mutant (**Fig. 8B, C**), while the period of free-running rhythms under LL conditions was slightly shortened in the *ef7* mutant (**Fig. 8D, E**). These results suggest that *Ef7* function might not be required for the clock function in the absence of light, but might mediate light input to the circadian clock in the presence of light.

Discussion

In this study, we identified and characterized *Ef7*, a rice ortholog of Arabidopsis *ELF3*, and demonstrated that it acts as a repressor of *Ghd7*, a key gene in the photoperiodic control of rice

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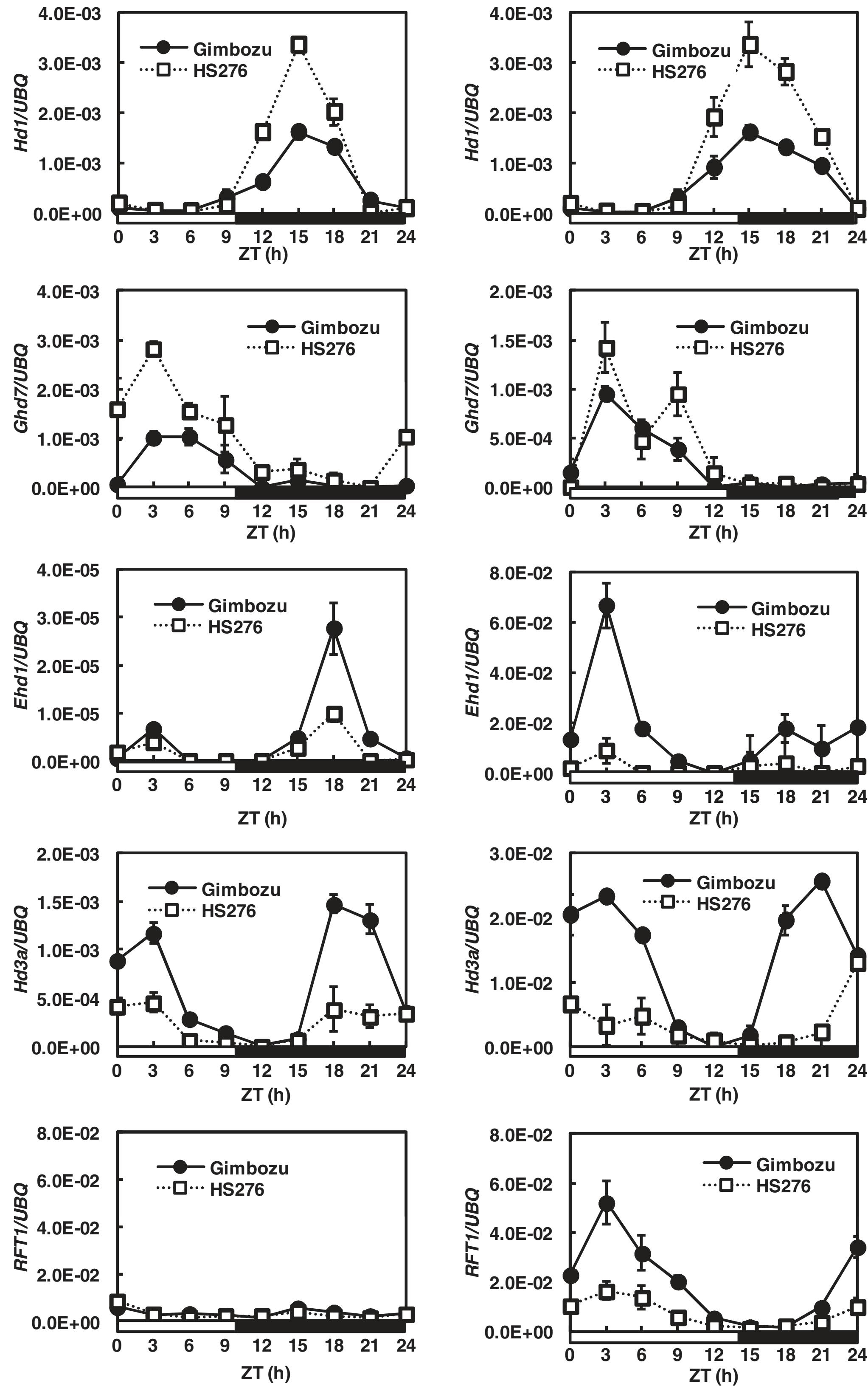

Fig. 5 Comparison of expression levels of *Hd1*, *Ghd7*, *Ehd1*, *Hd3a* and *RFT1* between the WT (Gimbozu) and the *ef7* mutant (HS276) under SD conditions (left) and LD conditions (right). Expression levels were normalized against expression of a ubiquitin (*UBQ*) gene. Error bars indicate standard deviations ($n = 3$). ZT, Zeitgeber time.

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flowering (**Fig. 9**). *Ghd7* is a major floral repressor under LD conditions: it strongly represses *Ehd1* expression and the genes downstream of *Ehd1*, such as *Hd3a* and *RFT1* (Xue et al. 2008, Itoh et al. 2010). Our results clearly show that the expression of *Ehd1* and *Hd3a* was down-regulated in the *ef7* mutant under both SD and LD conditions and the expression of *RFT1* was down-regulated in the *ef7* mutant under LD conditions (**Fig. 5**). This suggests that *Ef7* normally promotes flowering by repressing *Ghd7* expression. The expression of *Ef7* was strongly detected in mesophyll cells (**Fig. 4B–F**), but not detected in vascular tissue where the *Ef7* target gene, *Ghd7*, is expressed (Xue et al. 2008). This suggests the tissue non-autonomous action of *Ef7* on flowering regulation. The *ef7* mutant had long awns, few panicles and increased panicle length in addition to delayed flowering, and no other phenotypic differences were found (**Fig. 1**, **Supplementary Fig. S1B**). Complementation of the *ef7* mutant by a transgene expressing *OsELF3-1* (*Ef7*) also partially rescued the phenotypes of these traits (data not shown), indicating that *Ef7*, whether directly or indirectly, is involved in the awn length, number of panicles and panicle length in addition to flowering. It was reported that *Heading date 17* (*Hd17*), a QTL associated with a difference in flowering time between two Japanese rice cultivars 'Nipponbare' and 'Koshihikari', was located near *Ef7* (Matsubara et al. 2008), and that the difference in flowering time between alleles of *Hd17* appears to result from a single nucleotide polymorphism within a putative gene encoding a homolog of the Arabidopsis ELF3 protein (Matsubara et al. 2012). This evidence indicates that *Ef7* is identical to *Hd17*.

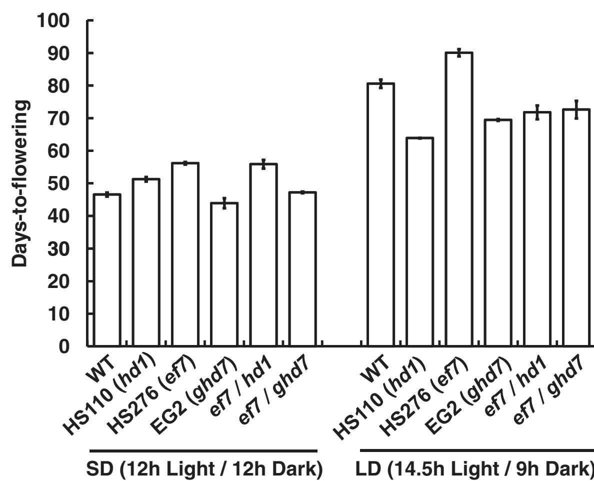

Fig. 6 Effect of the *ef7* mutant gene on flowering time in combination with defective *Ghd7* or *Hd1* alleles (*ghd7* and *hd1*, respectively) under SD and LD conditions. Values are means ($n = 10$). Error bars indicate standard deviations.

The *ELF3* gene in Arabidopsis plays key roles in the control of circadian rhythms, flowering time and plant morphology. *ELF3* encodes a plant-specific nuclear protein without any known functional domains (Hicks et al. 2001, Liu et al. 2001). We found five motifs in plant ELF3-like proteins that are highly conserved among both dicots and monocots (**Supplementary Fig. S2**). On the other hand, the candidate nuclear localization signal (NLS) sequence (Liu et al. 2001) is not conserved between Arabidopsis ELF3 and other ELF3-like proteins (**Supplementary Fig. S2**). In the rice Ef7 protein sequence, no candidate NLS region could be detected by the SignalP4.0 (http://www.cbs.dtu.dk/services/SignalP/, Petersen et al. 2011) or iPSORT (http://ipsort.hgc.jp/, Bannai et al. 2002) programs. In Arabidopsis, ELF3 interacts directly with PHYB, and an ELF3–PHYB complex regulates gene expression (Liu et al. 2001). In this study, expression of *Ghd7* and *Cab1R* in the *ef7* mutant was highly sensitive to white and red light (**Figs. 7**, **8**), suggesting that Ef7 has a function similar to ELF3 and might also accumulate in the nucleus to regulate gene expression via a complex with rice PHYB.

ELF3 in Arabidopsis regulates the timing of maximum responsiveness to light input into the circadian clock, a phenomenon commonly referred to as gating (Millar and Kay 1996,

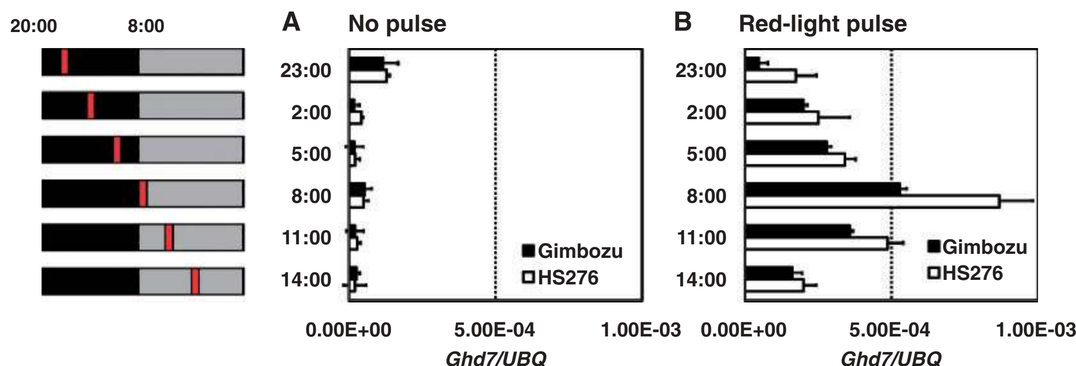

Fig. 7 Analysis of *Ghd7* gated expression levels in the WT (Gimbozu) and the *ef7* mutant (HS276). Both genotypes were entrained by 12 h light/12 h dark for 2 weeks, then transferred to darkness at dusk (Zeitgeber time = 20:00). Samples were exposed to a single 10 min red light pulse at various times and harvested 2 h after the beginning of exposure. Black boxes, night; gray, subjective day; red boxes, red light pulses. (A) *Ghd7* expression without red light pulse treatment. (B) *Ghd7* expression with red light pulse treatment.

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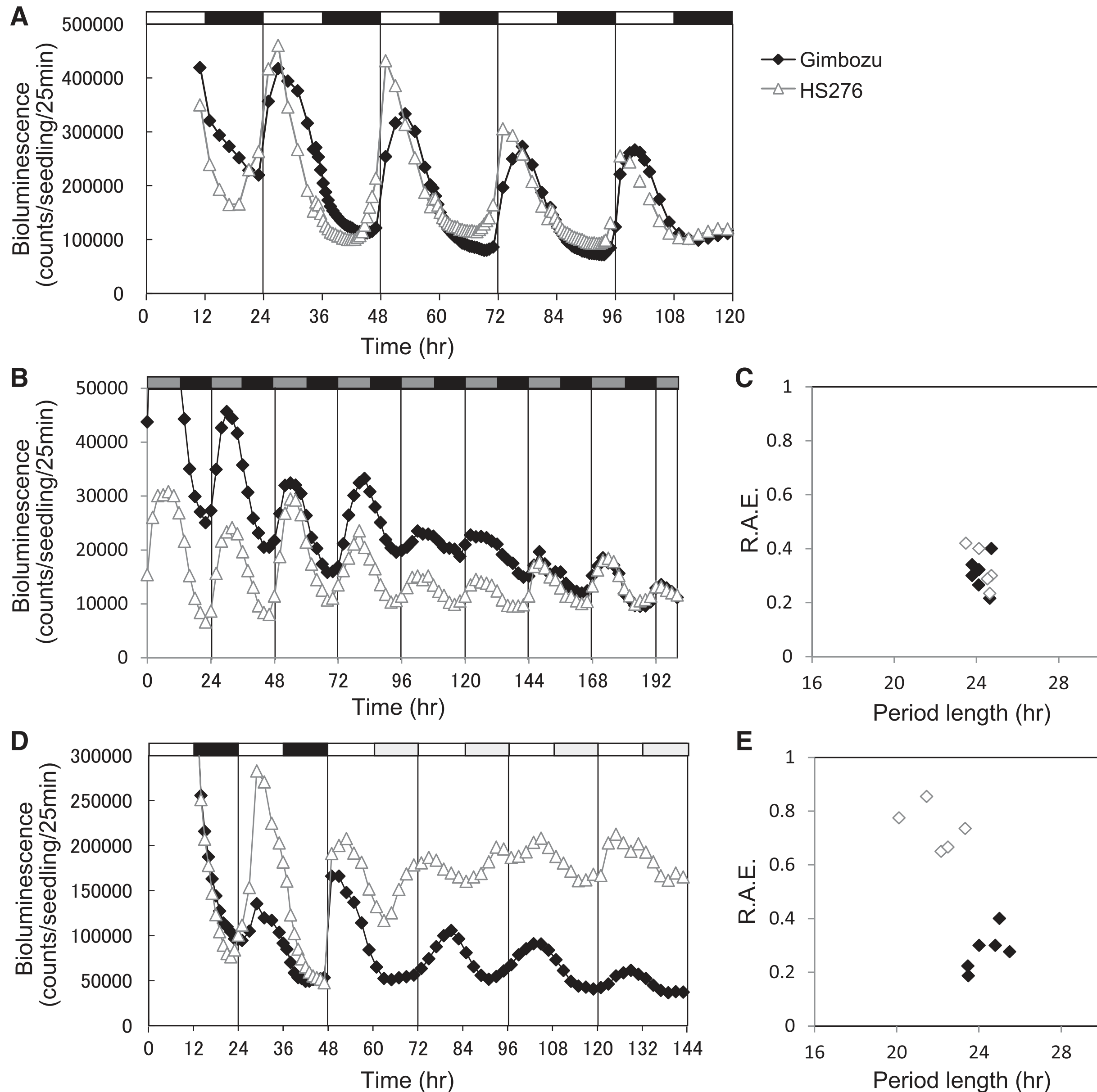

Fig. 8 (A, B, D) Bioluminescence analysis of *Cab1R:LUC* expression in plants exposed to (A) a 12 h light/12 h dark cycle, (B) constant dark (DD) or (D) constant light (LL). (C, E) Period lengths and relative amplitude errors (R.A.E.) in (C) DD and (E) LL, calculated by FFT-NLLS analysis according to data from 24 to 96 h after transfer of the plants to constant conditions. Seedlings were grown under light/dark cycles for 5 d before transfer to (B) DD [Zeitgeber time (ZT) 0] or (D) LL (ZT 48). White boxes, light; black, dark; gray, subjective light (B) or subjective dark (D) conditions.

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McWatters et al. 2000). The *elf3* loss-of-function mutation confers arrhythmia on clock-regulated rhythms of leaf movement, hypocotyl elongation and gene expression under LL but not under DD (McWatters et al. 2000, Covington et al. 2001, Hicks et al. 2001, Nusinow et al. 2011). In the *ef7* mutant, the period of free-running *Cab1R:LUC* expression was slightly shortened under LL conditions relative to the WT (**Fig. 8**). This shortened-period phenotype is different from the *elf3* mutant phenotype in Arabidopsis. Because two *ELF3* orthologs have been found in the rice genome (**Fig. 2C**; Miwa et al. 2006, Fu et al. 2009), the second *ELF3*-like gene, *OsEF3/OsELF3-2* (Os01g0566050), whose product shares 77% amino acid identity with the predicted product of *Ef7*, may also function and act redundantly to *Ef7*.

ELF3 has also been proposed to act as a transcriptional regulator that controls photoperiodic expression of clock-controlled genes, including flowering time genes (Liu et al. 2001). In Arabidopsis, the key to the photoperiodic flowering response is the activation of *FT* (Imaizumi and Kay 2006, Jaeger et al. 2006, Zeevaart 2006, Turck et al. 2008). Expression of *FT* is activated by *CO* (Koornneef et al. 1998, Onouchi et al. 2000, An et al. 2004). A circadian oscillator, such as *GI*, controls the

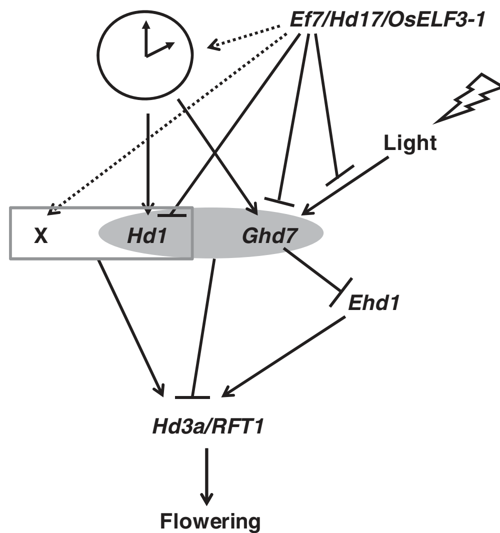

Fig. 9 Schematic representation of the roles of *Ef7/Hd17/OsELF3-1* in the gene regulatory network controlling photoperiodic flowering in rice. The gray oval and box indicate genetic interaction under LD and SD conditions, respectively. X indicates an unidentified gene(s).

diurnal rhythmic expression of *CO*, and the CO protein, which is stabilized by light input mediated by PHYB, induces *FT* expression (Putterill et al. 1995, Suarez-Lopez et al. 2001, Valverde et al. 2004, Imaizumi and Kay 2006). The N-terminal region of ELF3 interacts with the C-terminal region of PHYB (Liu et al. 2001). ELF3 blocks light input into the clock by inhibiting PHYB function. Thus, the *elf3* mutant accelerates flowering by activation of the GI–CO pathway under both SD and LD conditions (Yoshida et al. 2009). In rice, *Ghd7* expression is regulated by phytochrome signals and gating mechanisms (Itoh et al. 2010). The *ef7* mutant is more sensitive to red light than the WT, but the peak of gated *Ghd7* expression is unchanged. Therefore, we suggest that *Ef7* might block the light input into *Ghd7* by inhibiting the phytochrome signal, and that increased accumulation of the *Ghd7* transcript in the *ef7* mutant might cause the *ef7* late-flowering phenotype under both SD and LD conditions (**Fig. 9**).

Further, expression analyses demonstrated that *Ef7* also functions as the repressor of *Hd1* expression under both SD and LD conditions. *Hd1* functions as a floral promoter under SD conditions, while it functions as a floral repressor under LD conditions (Izawa et al. 2002, Hayama et al. 2003, Ishikawa et al. 2005). This functional conversion depends on phytochrome signaling (Izawa et al. 2002, Ishikawa et al. 2011). Hayama et al. (2003) demonstrated that increased expression of *Hd1* during the light period caused late flowering. Therefore, it is considered that the high expression of *Hd1* during the light period enhances the floral repression. Under LD conditions, *Hd1* expression was up-regulated during not only the dark period but also the light period around dusk in the *ef7* mutant. This suggests that this increased expression of *Hd1* is involved in delayed flowering in the *ef7* mutant. Genetic analyses suggest that the *Hd1* and *Ghd7* pathways might be interdependent. *Hd1* and *Ghd7* both encode CCT domain-containing proteins (Yano et al. 2000, Xue et al. 2008). It is therefore possible that these genes function as floral repressors acting on the same pathway or by interactions via other mediators, and that *Ef7* promotes flowering by negatively regulating the pathways of the *Ghd7* and *Hd1* gene products (**Fig. 9**). Under SD conditions, *Hd1* was up-regulated in the *ef7* mutant only during the dark period. It was therefore expected that this increased expression of *Hd1* could enhance the floral promotion. However, the *ef7* mutant showed delayed flowering under SD conditions. This observation implies the possibility that an unidentified gene(s), which interacts with *Hd1* to promote flowering under SD conditions, is regulated by *Ef7* (**Fig. 9**). Arabidopsis CO, a protein with a DNA-binding domain similar to the HEME ACTIVATOR PROTEIN 2 (HAP2) protein, interacts independently with HEME ACTIVATOR PROTEIN 3 (HAP3) and HEME ACTIVATOR PROTEIN 5 (HAP5), indicating that the CO–HAP3–HAP5 complex may promote flowering (Wenkel et al. 2006). *Hd1* is a rice ortholog of *CO* that also encodes a protein with a DNA-binding domain similar to that of HAP2 (Yano et al. 2000). This suggests that *Hd1* may form a complex with proteins encoded by rice orthologs of Arabidopsis *HAP3* and *HAP5*. Recently it was reported that *DTH8/Ghd8/Hd5*, which encodes a HAP3 subunit protein, delayed flowering under LD conditions but promoted it under SD conditions (Wei et al. 2010, Yan et al. 2011). In addition, an epistatic interaction between *Hd1* and *DTH8/Ghd8/Hd5* was demonstrated (Lin et al. 2003). Thus, a complex of Hd1–DTH8/Ghd8/Hd5–HAP5 might regulate flowering in rice. Further analyses will allow us to understand how *Ef7* functions to regulate photoperiodic flowering via the *Hd1* and *Ghd7* pathways.

Materials and Methods

Plant materials and growth conditions

The late-flowering mutant HS276 was induced by γ-irradiation of seeds of the *japonica* rice cultivar Gimbozu. The late flowering of HS276 is caused by the single recessive mutant gene *ef7* (Yuan et al. 2009). The HS110 mutant, which was similarly induced, harbors a defective mutant allele at the *Hd1* locus (Yano et al. 2000). EG2, which is commonly used as a tester line for studies of flowering time in rice, harbors a defective allele of *Ghd7* in the Gimbozu genetic background (Yamagata et al. 1986, H. Saito et al. unpublished results). HS276 was crossed with HS110 and EG2 to develop the *ef7/hd1* and *ef7/ghd7* double mutants, respectively. Plants were grown in a controlled-growth cabinet (Especmic TGEH-9) under SD conditions (12 h light/12 h dark or 10 h light/14 h dark) or LD conditions (14.5 h light/9.5 h dark) at 60% relative humidity. Light was provided by metal halide lamps (300–1,000 nm spectrum, photosynthetic photon flux density 500 μmol m^{-2} s^{-1}). Temperatures were 28°C in the light and 24°C in the dark.

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For evaluation under ND conditions, plants were also grown in a paddy field in Kyoto, Japan (35°00′N), beginning in mid-April. The date when the first panicle emerged from the sheath of the flag leaf was recorded for each plant and used to calculate days to flowering.

Cloning of the *ef7* mutant gene

Yuan et al. (2009) reported that *ef7* was located in a 129.5 kb region on the short arm of chromosome 6. We constructed a BAC clone library of HS276 and screened for BAC clones including the candidate region by using the two closest DNA markers, INDEL0399-6 and INDEL3784-2 (Yuan et al. 2009). We analyzed the sequence of the BAC clone and compared it with that of Nipponbare in RAP-DB (http://rapdb.lab.nig.ac.jp/: IRGSP build 5). To perform the complementation test of *ef7*, we cloned an approximately 9.4 kb genomic fragment of Gimbozu including the *Ef7* region, which was then digested by *Xho*I and *Bam*HI and cloned into the pPZP2H-lac binary vector (Fuse et al. 2001). The resultant plasmid was then introduced into the *ef7* mutant by means of *Agrobacterium*-mediated transformation (Toki et al. 2006). Homozygous T_2 progeny derived from single-copy T_0 transformants were grown under LD conditions, and their flowering time was recorded. Putative transgene-containing plants were selected on medium containing hygromycin, and copy number was estimated by TaqMan quantitative RT–PCR (qRT–PCR) (**Supplementary Table 2**).

Construction of *Ef7* phylogenetic tree

The evolutionary history was inferred using the Neighbor–Joining method. The optimal tree (sum of branch lengths = 4.68) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths proportional to the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2.25). The analysis involved 18 ELF3-like amino acid sequences, with Arabidopsis sequence AT3G21320 as an outgroup. All positions containing gaps and missing data were eliminated. There were a total of 409 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

GUS staining experiments

To generate pMLH7133GUS containing the fusion of the *Ef7* promoter with the GUS gene, DNA fragments containing the upstream region of *Ef7* (−2,000 to +3) conjugated at the *Hin*dIII and *Bam*HI sites at the 5′ and 3′ ends, respectively, were amplified by PCR. The PCR-amplified fragments were subcloned and sequenced. After digestion with *Hin*dIII and *Bam*HI, the DNA fragments were inserted into the *Hin*dIII–*Bam*HI site on the pMLH7133 vector (Mitsuhara et al. 1996). Plasmid pMLH7133GUS was used for transformation into WT rice plants as described above.

qRT–PCR analysis of gene expression

Total RNA was extracted from leaves by using the SDS–phenol method (Shirzadegan et al. 1991). Total RNA (2.5 or 3 μg) was primed with the dT^{18} primer by using Superscript II Reverse Transcriptase (Invitrogen), in accordance with the manufacturer's instructions. qRT–PCR analysis was performed by the TaqMan qRT–PCR method using an ABI7900HT (Life Technologies Inc.). cDNA corresponding to 50 ng of total RNA was used as the template for each TaqMan qRT–PCR. At least three PCRs using each template were performed to obtain an average value for the expression level. The PCR conditions were 10 min at 95°C, followed by 45 cycles of 15 s at 95°C, 60 s at 65°C and 1 s at 72°C. To quantify the expression of *Ghd7*, *Hd1*, *Ehd1*, *Hd3a*, *RFT1*, *Ef7* and *UBQ* (internal control), we used the specific primers and probes listed in **Supplementary Table S2**. For copy number standards, quantified fragments of cloned cDNA were used. The results are presented as means of at least two biological replicates, with three technical repeats for each biological replicate.

Analysis of light-induced expression of *Ghd7*

The WT (Gimbozu) and the *ef7* mutant (HS276) were grown under 12 h light/12 h dark conditions for 2 weeks to entrain the light/dark cycle. Then, they were transferred to darkness at dusk (Zeitgeber time = 20:00). Samples were exposed to a single 10 min red light pulse (the intensity was 12.5 μmol m^{-2} s^{-1}: Sanyo Electric Co. Ltd.) at various times and harvested 2 h after the beginning of exposure. RNA extraction and qRT–PCR were similar to those described above.

Bioluminescence assays of circadian rhythm

Construction of the *Cab1R:LUC* reporter construct was described in Matsubara et al. (2012). The generated binary plasmid was introduced into *Agrobacterium tumefaciens* strain E101 and transformed into Gimbozu and HS276 (Toki et al. 2006). After selection by bioluminescence, a few T_0 seedlings were grown in 3 ml of Murashige and Skoog medium (Murashige and Skoog 1962) in a 50 ml Falcon tube (Becton-Dickinson and Company) and entrained for 5 d under 12 h light/12 h dark at 30°C. The light source was a mixture of red and blue light-emitting diode lamps (Sanyo Electric Co. Ltd.) with a photon flux density of 200 μmol m^{-2} s^{-1}. An Aquacosmos photon-counting system (Hamamatsu Photonics Co. Ltd.) was used for all bioluminescence imaging experiments. Periods and relative amplitude errors were analyzed by the Fourier transform–non-linear least squares method (FFT-NLLS) in the BRASS interface (Plautz et al. 1997).

Supplementary data

Supplementary data are available at PCP online.

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