Ef7 Encodes an ELF3-like Protein and Promotes Rice Flowering by Negatively Regulating the Floral Repressor Gene Gh7 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 Gh7, 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 Gh7 allele provided further evidence that the delayed flowering of ef7 is mediated through the Gh7 pathway. The analysis of light-induced expression of Gh7 revealed that the ef7 mutant was more sensitive to red light than the wild-type plant, but the gate of Gh7 expression was unchanged. Thus, our results show that Ef7 functions as a floral promoter by repressing Gh7 expression under both SD and LD conditions.

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

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

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, Tsujii 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 photoperiodic 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 63.6, 87.7 and 100.0 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).
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.

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) Phylogenetic relationship of ELF3-like proteins from various plant species. Gene IDs and locus number are shown in Supplementary Fig. S2.
Os\textsubscript{06g0142600} (Os\textsubscript{ELF3-1}) is predicted to encode a 760 amino acid protein and is an ortholog of the Arabidopsis flowering time gene \textit{ELF3}; the predicted Os\textsubscript{ELF3-1} protein sequence is 38.3\% identical to that of Arabidopsis \textit{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 \textit{ef7} mutant phenotype was caused by the lesions in the ELF3-like protein, we complemented \textit{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 \textit{OsELF3-1} and a 2.1 kb downstream sequence. \textit{T1} and \textit{T2} plants containing this segment clearly showed earlier flowering than the \textit{ef7} mutant (Fig. 3). These results confirm that \textit{Ef7} encodes an ELF3-like protein.

Expression pattern of the \textit{Ef7} gene

We investigated the diurnal expression of the \textit{Ef7} gene in the WT and the \textit{ef7} mutant. In the WT, \textit{Ef7} expression started to increase after midnight, peaked just at dawn and gradually decreased during the daytime under SD conditions. In the \textit{ef7} mutant, the expression followed a similar pattern but was significantly lower at most time points (Fig. 4A). We carried out \textit{Ef7} promoter::\textit{GUS} (\(\beta\)-glucuronidase) reporter analysis to profile the \textit{Ef7} expression. \textit{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 \textit{ef7} mutant under SD and LD conditions

To identify potential downstream genes regulated by \textit{Ef7}, we examined the expression levels of five flowering-related genes (\textit{Hd1}, \textit{Ghd7}, \textit{Ehd1}, \textit{Hd3a} and \textit{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 \textit{Ehd1} and \textit{Hd3a} expression were lower in the \textit{ef7} mutant than in the WT, but \textit{RFT1} expression did not show any obvious difference between the two genotypes (Fig. 5). On the other hand, the peaks of \textit{Hd1} and \textit{Ghd7} expression were higher in the \textit{ef7} mutant than in the WT (Fig. 5). Under LD conditions, the peaks of \textit{Ehd1}, \textit{Hd3a} and \textit{RFT1} expression were lower in the \textit{ef7} mutant than in the WT, while those of \textit{Hd1} and \textit{Ghd7} were higher in the \textit{ef7} mutant than in the WT (Fig. 5). \textit{Ghd7} acts as a repressor of \textit{Ehd1} expression, and thereby delays flowering under LD conditions (Xue et al. 2008, Itoh et al. 2010). Our results therefore suggest that \textit{Ef7} negatively regulates the expression of \textit{Ghd7} and \textit{Hd1}, and consequently up-regulates the expression of the genes downstream of \textit{Ghd7} (i.e. \textit{Ehd1} and \textit{FT}-like genes) under both SD and LD conditions.
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, Covington 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 (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
Fig. 5 Comparison of expression levels of Hdl1, 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.
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.

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,
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 elf7 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, OsELF3/OsELF3-2 (Os01g0566050), whose product shares 77% amino acid identity with the predicted product of elf7, may also function and act redundantly to elf7.

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 Gl, controls the

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.
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 downstream 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 causes 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 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/gd7 double mutants, respectively. Plants were grown in a controlled-growth cabinet (Specmic 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.
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 XhoI and BamHI and cloned into the pPZP2H-lac binary vector (Fuse et al. 2001). The resultant plasmid was then introduced into the 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 modeled with a gamma distribution (shape parameter = 2.25).

The analysis involved 18 ELF3-like amino acid sequences, with the evolutionary history was inferred using the Neighbor–Joining method (Shirzadegan et al. 1991). 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.) was used for all bioluminescence imaging experiments. A n 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).

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 T₀ 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.

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 HindIII and BamHI sites at the 5′ and 3′ ends, respectively, were amplified by PCR. The PCR-amplified fragments were subcloned and sequenced. After digestion with HindIII and BamHI, the DNA fragments were inserted into the HindIII–BamHI 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 dT¹⁸ 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 ABI 7900HT (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 UBX (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.

Supplementary data

Supplementary data are available at PCP online.
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**References**


Hd3a promotes rice flowering


