Se14, Encoding a JmjC Domain-Containing Protein, Plays Key Roles in Long-Day Suppression of Rice Flowering through the Demethylation of H3K4me3 of RFT1

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Abstract

Floral transition from the vegetative to the reproductive growth phase is a major change in the plant life cycle and a key factor in reproductive success. In rice (Oryza sativa L.), a facultative short-day plant, numerous flowering time and flower formation genes that control floral transition have been identified and their physiological effects and biochemical functions have been clarified. In the present study, we used a Se14-deficient mutant line (HS112) and other flowering mutant lines to investigate the photoperiodic response, chromosomal location and function in the photoperiod sensitivity of the Se14 gene. We also studied the interactive effects of this locus with other crucial flowering time genes. We found that Se14 is independent of the known photoperiod-sensitive genes, such as Hd1 and Ghd7, and is identical to Os03g0151300, which encodes a Jumonji C (JmjC) domain-containing protein. Expression analysis revealed that the expressions of RFT1, a floral initiator known as a "florigen-like gene", and Ehd1 were up-regulated in HS112, whereas this up-regulation was not observed in the original variety of 'Gimbozu'. ChIP assays of the methylation states of histone H3 at lysine 4 (H3K4) revealed that the trimethylated H3K4 in the promoter region of the RFT1 chromatin was significantly increased in HS112. We conclude that Se14 is a novel photoperiod-sensitivity gene that has a suppressive effect on floral transition (flowering time) under long day-length conditions through the modification of chromatin structure by H3K4me3 demethylation in the promoter region of RFT1.

Introduction

Floral transition from the vegetative to the reproductive growth phase is a major change in the plant lifecycle that is critical to reproductive success. In rice (Oryza sativa L.), a facultative short-day plant, floral transition is promoted under short day-length conditions and delayed under long day-length conditions. To date, numerous flowering time and flower formation genes that control the floral transition of rice have been identified [1–4], and the physiological effects and biochemical functions have already been identified for many of these genes. Based on their physiological effects and interactions with other genes, several genetic pathways to floral transition have been proposed [5–7].

* Early heading date 1 (Ehd1) functions as a flowering time activator under both short and long day-length conditions [2], and regulates the duration of the basic vegetative growth period [9]. Ehd1 promotes the expression of two florigen-like genes, Heading date 3a (Hd3a) and Rice FT-like 1 (RFT1), and thereby accelerates flowering. Under long day-length conditions, the expression of Ehd1 is suppressed by Grain number, plant height and heading date 2 (Ghd7), which encodes a CCT (CO, CO-like, and TIMING OF CAB1)-domain protein [4]. Ghd7 acts as a key factor in the photoperiodic pathway to flowering and delays flowering under long day-length conditions through inhibiting Ehd1 expression.

Under long day-length conditions, Ghd7 is up-regulated by gated signaling of red light [9]. We previously reported that Photoperiod sensitivity-13 (Se13) encodes phytochromobilin synthase involved in phytochrome activity [10]. The Se13-deficient mutants flowered extremely early (about 40 days earlier than the original variety) even under long day-length conditions due to the lack of functional phytochromes (Yoshitake et al., unpublished). Such a red-light-dependent suppression pathway(s), including Ehd1 and Ghd7, does not exist in Arabidopsis and is considered specific to rice. In addition to Ghd7, OsMADS50 and Ehd2 are known to be activators of the florigen-like genes Hd3a and RFT1 through the up-regulation of Ehd1 expression, thereby promoting flowering under long day-length conditions [3,11]. On the other hand, Heading date 1 (Hd1) is another key regulator that controls Hd3a expression in the photoperiodic pathway, independent of Ehd1, and delays and slightly promotes flowering under long and short day-length conditions, respectively [12,13].

Chromatin structure allows accessibility of factors and cofactors that regulate gene expression for biological processes of plant development including floral transition. Histone lysine methylation...
is an essential epigenetic modification of chromatin structure having activating and suppressing effects on gene expressions [14]. The methylation of histone H3 lysine 4 (H3K4) and lysine 36 (H3K36) primarily has an activating effect on gene expression, whereas that of histone H3 lysine 9 (H3K9), lysine 27 (H3K27), and histone H4 lysine 20 (H4K20) has a suppressing effect on gene expression [15].

It has been suggested that Jumonji C (JmjC) domain-containing proteins function as histone demethylases [16]. These proteins are capable of demethylating all of the mono-, di- and trimethylated lysines of histones [17]. Recent studies on Arabidopsis thaliana, a facultative long-day plant, have shown that the expression of FLOWERING LOCUS C (FLC) and FLOWERING LOCUS T (FT) are regulated through chromatin modifications. FLC is epigenetically regulated by ARABIDOPSIS TRITHORAX 1 (ATX1) and the EARLY FLOWERING IN SHORT DAYS (EFS) which mediate H3K4 and H3K36 methylation, respectively, and act as inhibitors of Arabidopsis flowering [18–20]. Arabidopsis thaliana Jumonji 4 (Jmj4) and EARLY FLOWERING6 (ELF6), both of which encode JmjC-domain containing proteins, function as H3K4 demethylases of FT histones and repress FT expression [21].

In rice, JmjC domain-containing proteins are also conserved and some of these have been identified. JMJ706, a rice member of the JMJD2 family of JmjC genes [17], is involved in H3K9 demethylation, which is required for the expression of a subset of regulatory genes for rice floral development [22]. JMJ703 is a histone H3K4-specific demethylase and is responsible for a unique mechanism of controlling retrotransposon activity, which further strengthens the link between epigenetic silencing and genome stability [23].

Our previous study indicated that the early flowering of a mutant line HS112 was conferred by a single recessive mutant gene, photoperiod sensitivity-14 (se14) [24]. In the present study, we investigated the photoperiodic response, chromosomal location and function of the se14 locus, including the interactive effects of this locus with other crucial flowering time genes. We found that the functional allele se14 at the Se14 locus is a unique photoperiod-sensitivity gene that encodes a JmjC domain-containing protein with a zinc-finger (ZnF) domain, and functions as a demethylase of H3K4 methylation of the RFT1 chromatin, resulting in delayed flowering under long day-length conditions.

Results

Analysis of Photoperiodic Response

HS112 is an early flowering time mutant, which was induced by gamma-ray irradiation of seeds of the japonica rice variety Gimbozu (WT; Figure 1A). The early flowering of this mutant line has proved to be conferred by a single recessive mutant allele se14 at the Se14 locus [24]. We examined the days to heading (DH; = flowering time) of HS112 and the WT under the following five day-length conditions: 10 h (SD), 14.5 h (14.5 LD), 16 h (16 LD), 24 h (24 LD), and a natural day-length (ND) in our experimental field in Kyoto, Japan (NL35°01'). In addition, we examined the DH of two single mutant lines, hd1 and ghd7, which harbor recessive mutant alleles at the Hd1 and Ghd7 loci, respectively, under 16 LD and 24 LD conditions. Under SD, HS112 (54.9 DH) showed almost the same DH as that of WT (59.8 DH). Under 14.5 LD, however, HS112 exhibited 75.7 DH, while WT (91.8 DH) flowered two weeks later than HS112 (Figure 1B). Under ND, both HS112 and WT exhibited similar DH to plants under 14.5 LD, respectively. In Kyoto, the natural day-length is longer than the critical day-length (approximately 13.5 h) and does not exceed 14.5 h during the cropping season from May to the end of July [25]. This is regarded as a long day-length condition. After July, it becomes shorter than 13.5 h, which is regarded as a short day-length condition [10]. Thus, the ND and 14.5 LD used in this study were considered to be almost the same photoperiod. Under 16 LD and 24 LD conditions, neither the WT nor HS112 flowered even after 150 days from sowing (Figure 1C). In contrast, the hd1 and ghd7 mutants flowered 115 and 130 days after sowing, respectively. These results indicate that the Se14 locus is involved in the photoperiodic pathway to flowering, but the role of the Se14 locus is different from the roles of the Hd1 and Ghd7 loci.

Identification of Chromosomal Location and the Candidate Gene of Se14

We attempted to perform a fine mapping of the Se14 locus using the F2 population, comprised of 96 plants, from a cross between HS112 and a chromosome segment substitution line SL13 harboring a Kasalath-derived chromosome segment including the candidate region of the Se14 locus on the short arm of chromosome 3, on a Nipponbare background [26]. Experimental
results demonstrated that the Se14 locus was located in a region with a physical distance of approximately 1 Mb between the two markers, RM14388 and MK3_6, on chromosome 3. Further analysis was conducted using 3920 F3 plants from the recombinants (F3 plants) of RM14388 and MK3_6, with five additional SSR markers and one INDEL marker. Consequently, the Se14 locus was narrowed down to the region between the two SSR markers, RM14394 and RM14395, with a physical distance of less than 46 kb (Figure 2B). According to the Rice Annotation Project Database (RAP-DB; http://rapdb.lab.nig.ac.jp), this 46 kb genomic region contains 14 genes supported by full-length cDNA. We analyzed the nucleotide sequences of these regions of WT and HS112, and found no mutations in HS112 except for a 23 bp deletion at Os03g0151300, which encodes a Jumonji C (JmjC) domain-containing protein. This deletion was located in the first exon, resulting in a frame-shift mutation spoiling all the functional domains by producing a premature stop codon (Figure 2C). It is therefore considered that Os03g0151300 is a likely candidate for the Se14 gene. To determine the cDNA sequence, we performed 5’-RACE and 3’-RACE experiments using Os03g0151300-specific primers. Interestingly, the experimental results showed that in addition to the cDNA sequence of Os03g0151300, the transcript included four additional exons whose sequences were partially identical with those of the cDNA of Os03g0151400, located next to Os03g0151300 (Figure 2D). The combined cDNA encodes a protein containing two functional domains, Jumonji N (JmjN) and JmjC, with four copies of C2H2-type zinc finger (ZnF) domains, which share high homology with Arabidopsis ELF6 (Figure 2D and Figure S1). This indicates that Se14 is most likely Os03g0151300, and the spoiling of functional domains due to the frame-shift mutation of Os03g0151300 causes the decrease of the photoperiod sensitivity of HS112.

Analysis of Interactions of the Se14 Locus with the Ehd1, Se13, Hd1 and Ghd7 Loci

Interactions of the Se14 locus with other flowering time loci were investigated using four single mutant lines, ehd1, hd1, ghd7 and se13, and four double mutant lines, se14 ehd1, se14 hd1, se14 ghd7 and
se14 se13. These lines were grown under ND conditions in Kyoto. The double mutant lines, se14 hd1 and se14 ghd7, flowered earlier than their respective single mutant lines (Figure 3A and 3B). In addition, the double mutant lines, se14 ehd1, flowered independently between their respective single mutant lines (Figure 3C). This indicates that the functional allele Se14 at the Se14 locus suppresses flowering independently of Ehd1, Hd1 and Ghd7. On the other hand, there was no significant difference in DH between the double mutant line se13 se14 and its single mutant line se13, suggesting that the functional allele Se14 does not affect flowering time in an Se13-deficient genetic background (Figure 3D). Se13 encodes phytochromobilin synthase, which is involved in phytochrome activity [10], and the se13 mutant flowered extremely early under long day-length conditions. Therefore, Se14 might be involved in the suppression pathway regulated by the red-light signal.

Expression Analyses of Flowering Time Genes in HS112

The diurnal expression of flowering time genes under a 14.5 h day-length condition (a long day-length condition) was analyzed to elucidate the molecular regulation of early flowering of HS112 conferred by se14. The expression of Ehd1 was increased in HS112 at night, but this increase was not observed in the WT (Figure 4A). The expression of RFT1 was increased in HS112, except during early night, while the WT showed lower expression almost throughout the day (Figure 4B). The expression of Hd3a was somewhat elevated during daytime in HS112, but there was no significant difference between HS112 and the WT (Figure 4C). On the other hand, no significant difference was observed in the expression of Ghd7 and Hd1 between HS112 and the WT (Figure 4D and 4E). Interestingly, there was no apparent difference in the expression of OsMADS50 and Ehd2, both of which are positive regulators of Ehd1, between HS112 and the WT (Figure 4F and 4G). These results indicate that the Se14 directly or indirectly suppresses the expression of RFT1 and Ehd1.

Investigation of H3K4 Methylation States in HS112

Histone methylation marks are generally associated with transcriptional chromatin states. According to the predicted amino acid sequence, the Se14 protein is expected to function as an H3K4 demethylase. To confirm this, we investigated the deposition of histone methylation marks on the chromatin of Ehd1, Hd3a and RFT1 by chromatin immunoprecipitation (ChIP) assays covering every 500 bp region 2 kb-upstream of the transcription start site (TSS) and the coding region (Figure 5A). Plants of HS112 were grown under the same experimental conditions as those in expression analysis, 14.5 h day-length (14.5 h light, 30°C/9.5 h dark, 25°C) at 70% relative humidity. Thirty days after sowing, we collected fully opened leaves from the top of seedlings. Experimental results showed that the H3K4me3 levels were significantly increased in the II and III regions of the RFT1 chromatin in HS112, while those were slightly increased in the coding region (Figure 5D). In coding region of RFT1, the H3K4me3 levels were slightly increased (Figure 5D). On the other hands, the H3K4me3 level in the chromatin regions including the both of promoter and coding regions of Ehd1 and Hd3a did not significantly differ between HS112 and the WT (Figures 5B and C). These results suggest that Se14 functions as a demethylase of the H3K4 tri-methylation mark in the RFT1 chromatin region.

Discussion

In this study, we confirmed that the early flowering of the mutant line HS112 is caused by a single recessive mutant gene se14 at the photoperiod sensitive locus Se14. Analysis of photoperiodic response showed that the Se14 locus is involved in the photoperiodic pathway in rice, but the role of this locus differs from the roles of two other crucial loci in the photoperiodic pathway, Hd1 and Ghd7. Subsequent linkage analysis revealed that the Se14 locus is between RM14394 and RM14395, in a region with a physical distance of less than 46 kb on chromosome 3 (Figure 2B). A
database search for this region showed that Os03g0151300 is a candidate for the Se14 gene. As a result of sequence analysis of the candidate region, HS112 was found to harbor a 23 bp deletion in exon 1 of Os03g0151300, which causes a frame-shift mutation producing a premature stop codon in all of the functional domains (Figure 2C). Therefore, we conclude that the Se14 locus is identical to the Os03g0151300 locus, and se14 is a loss-of-function allele at the Se14 locus. The Se14 gene encodes a JmjN and JmjC domain-containing protein with four copies of the C2H2-type ZnF domain. ChIP assays showed that trimethylated H3K4 of HS112 was specifically increased at the upstream region of the RFT1 chromatin (Figure 5D). Thus, Se14 is considered to be a key gene in controlling H3K4 trimethylation states in the RFT1 chromatin, thereby acting as a suppressor of RFT1 under long day-length conditions.

Chromatin structure is important for the accessibility of factors and cofactors that regulate gene expressions. Histone lysine methylation has garnered a lot of attention due to the complicated role it plays in the epigenetic modification that both activates and suppresses gene expression [14]. The methylation state of H3K4 primarily has an activating function of gene expression involved in flowering time control [15, 27]. Recent studies on Arabidopsis have suggested that JmjC domain-containing proteins function as histone demethylases [16]. They are capable of demethylating all of the mono-, di- and tri-methylated lysines of histones [17]. It has been reported that Atjmj4 and ELF6, which encode a JmjC-containing protein resembling those of the human JARID1 family, function as H3K4 demethylases of FT histones and suppress FT expression [21]. Yang et al. (2012) reported that overexpression of JMJ15, which is a member of the H3K4me3 demethylase JARID1 family, resulted in obvious early flowering due to the reduction in H3K4me3 at the FLC locus associated with the suppression of FLC transcription levels [28]. It is therefore believed that JmjC domain-containing proteins are crucial factors for mediating the H3K4me state and regulating gene expression involved in flowering time control.

Chen et al. (2013) demonstrated that, in rice, a loss-of-function mutation of a JmjC domain-containing protein, ELF6, affected stem elongation and plant growth, which may be related to the increased expression of cytokinin oxidase genes due to failure of demethylation of H3K4me marks [29]. Therefore, the H3K4me mark is important for the epigenetic remodeling of the chromatin structure that regulates rice growth. Genome-wide analyses of H3K4 methylation states in Arabidopsis and rice showed that H3K4me marking are found to be almost exclusively genic, and that H3K4me2 and H3K4me3 accumulate predominantly in promoters and 5′ genic region [30, 31]. On the other hand, Li et al (2008) reported that 25.3% of H3K4me2 and 27.2% of H3K4me3 were found in the region without a gene body (annotated transcribed region) plus its putative promoter (1 kb region upstream of the annotated transcription start site [TSS]) [32]. In human, it is reported that H3K4 states at the 1 kb upstream region from TSS is strongly associated with gene expression [33]. Therefore, we concluded that the high H3K4me3 level over 1 kb upstream region (position II and III in Figure 5) from TSS of RFT1 is essential to up-regulate the expression. Our study is the first to demonstrate that JmjC domain-containing proteins regulate the H3K4me state associated with the expression of
RFT1. Thus, the epigenetic modification of JmjC domain-containing proteins based on the H3K4me state is a key regulatory system for flowering time control in rice.

It is noteworthy that the H3K4me3 states of Ehd1 and Hd3a in HS112 were not different from those of the WT (Figure 5). This indicates that the Se14 demethylation is specific to the H3K4me state of RFT1. Ehd1 and RFT1 are paralogs and members of the phosphatidylethanolamine-binding protein gene family [34]. The function of the two paralogs, however, appears to have diverged after duplication [35]. It has been shown that Ehd1 and RFT1 are the major activators of flowering under short and long day-length conditions, respectively [36–38]. Furthermore, the nucleotide sequences of RFT1, compared with those of Hd3a, are highly diversified among varieties, suggesting that RFT1 has diverged more rapidly than Hd3a during rice evolution [35]. Taken together, it appears that the selectivity based on histone modification by Se14 might have different regulatory actions dependent upon photoperiod, resulting in the long-day suppression of flowering.

Analyses of interallelic interactions and gene expression indicated that Se14 regulates flowering time independently of Hd1 and Ghd7 (Figures 3 and 4). Ioh et al. (2010) demonstrated that Ghd7 is regulated by gated phytochrome signaling and repressed the expression of Ehd1 [9]. In present study, the expression of Ehd1 was up-regulated in HS112, in spite of high expression of Ghd7. In addition, the H3K4 tri-methylation states of Ehd1 were not changed in HS112. Therefore, Se14 might be indirectly involved in the repression of Ehd1 independent with the pathways of Ghd7 (Figure 6). Further, the analysis of interallelic interaction between Se14 and Ehd1 suggested that Se14 represses flowering independently of Ehd1. Thus, it is indicated that Se14 is more effective to delay flowering through the repression of RFT1 by H3K4me3 demethylation than the indirect repression of Ehd1. On the other hands, the analysis of interallelic interaction between Se14 and Hd3a demonstrated that Se14 did not function in the Hd3a-deficient genetic background (Figure 3C). Interestingly, the expression of Se14 was down-regulated in the Hd3a-deficient genetic background (Figure S2). Jang et al. (2011) demonstrated that the repression of phyA by light is correlated with alternations in specific histone marks [39]. In the dark, methylation of H3K4me3 were detected at the active phyA locus, whereas upon light treatment, increased H3K27me3 mark and decreased H3K4me3 mark were associated with the repressed gene [39]. They also reported that the chromatin modifications were blocked in the phyB-9 mutant in red light, indicating that these changes are mediated by the phyB signaling pathway [39]. Our study also showed that the diurnal expression of Se14 was highly transcribed during the light period (Figure S2). It is therefore suggested that the Se14 might be mediated by the red-light signal (Figure 6). Further analysis is necessary to clarify the light-mediated H3K4me3 demethylation.

Red-light signaling via phytochromes is the most important factor to repress flowering under LD condition. Osugi et al. (2011) showed that phytochromes not only regulate Ghd7 transcription but also affect Ghd7 protein activity [40]. In addition to the Ghd7 protein, the Hd1 protein is known to act as a suppressor of Hd3a
under long day-length conditions [12,41,42]. Ishikawa et al. (2011) reported that the inhibitory effect of the Hd1 protein on H3K4me expression is dependent on phyB [43]. Ishikawa et al. (2011) also suggested that the phyB-mediated Hd1 suppression of H3K4me expression is a component of the molecular mechanism for critical day length in rice [43]. Our results have let us propose a novel flowering repression pathway mediated by red-light signaling. We believe that this pathway of Se14 will yield crucial information for understanding the daylength dependent repression of flowering in rice.

Materials and Methods

Plant Materials

A total of eleven variety/lines, Gimbozu (WT), HS112 (se14), HS169 (ehd1), HS110 (hd1), X61 (se13), EG2 (ghd7), ehd1 se14, hd1 se14, se13 se14, ghd7 se14 and SL13, were used. The WT is the japonica rice variety Gimbozu. HS112 is an early flowering time mutant line, which was induced by gamma-ray irradiation of WT seeds, and its early flowering has been proven to be caused by a single recessive mutant gene se14 at the Se14 locus on chromosome 3 [24]. ehd1, hd1 and se13 are also flowering time mutant lines, which were created by gamma-ray irradiation of WT seeds, and their differential flowering time is conferred by single recessive mutant alleles, ehd1, hd1 and se13, at the Ehd1, Hd1 and Se13 locus, respectively [13,18]. ghd7 is a tester line for studying flowering time of rice, and its early flowering is controlled by a single recessive allele ghd7 at the Ghd7 locus in the Gimbozu genetic background [44]. ehd1, hd1 se14, se13 se14, and ghd7 se14 are double recessive mutant lines, which were developed from crosses of HS112 with ehd1, hd1, se13 and ghd7, respectively, and harbor ehd1, hd1, se13, and ghd7 in addition to se14, respectively. SL13 is a chromosome segment substitution line (CSSL) harboring a Kasalath-derived chromosome segment including the candidate region of the Se14 locus on chromosome 3 on the Nipponbare background (Figure 2A, [26], and the Rice Genome Center: http://www.rgrc.dna.afrc.go.jp). Kasalath is an indica rice variety.

Analysis of Photoperiodic Response

Three single mutant lines, HS112 (se14), hdl, ghd7 and WT were used. Ten seeds of each line were sown on field soil filled in a 3.6-L pot and covered with granulated soil. Seedlings were thinned to 5 plants per pot 14 days after sowing, and were grown under four artificial day-length conditions at the green house in Kyoto University, Kyoto, Japan (35°01’N, 135°46’E); 10 h (SD: short day-length), 14.5 h (14.5 LD:14.5 h long day-length), 16 h (16 LD:16 h long day-length) and 24 h (24 LD: continuous light). We also cultivated each plant under natural day-length conditions (ND) at the green house in Kyoto University. In Kyoto, the natural day-length is longer than the critical daylength (approximately 13.5 h) during the cropping season from May to the end of July [25], regarded as a long photoperiod condition, and after that it becomes shorter than 13.5 h regarded as a short photoperiod condition [10]. In addition to natural day length (8:00–18:00), supplementary artificial light from incandescent lamps (3.24 Wm−2 at soil surface) was used for the 14.5 LD, 16 LD, and 24 LD day-length treatments. The experiment was conducted from 21 May to late October, 2012, with two replications. Flowering time was recorded for each plant when the first panicle emerged from the sheath of the flag leaf.

Identification of Chromosomal Location and the Candidate Gene of Se14

Our previous study showed that the Se14 locus was located between two markers, RM14351 and MK3_6, on chromosome 3 [24]. To narrow down the candidate region of the Se14 locus, the F2 population, comprising 96 plants from the cross between HS112 and SL13, was grown in a paddy field in Kyoto in 2012. We conducted a fine mapping of the Se14 locus with SSR (simple sequence repeat) markers and mPing sequence characterized amplified region (SCAR) markers [45]. mPing SCAR markers are based on polymorphisms of the transposable element mPing insertion sites. To further narrow down the region of the mutant gene, we used five SSR markers and one insertion and deletion (INDEL) marker. Flowering time was recorded for each plant when the first panicle emerged from the sheath of the flag leaf. To identify the full-cDNA sequence of Se14, 5’-RACE and 3’-RACE were performed with the 5’-full RACE Core Set and the 3’-full RACE Core Set (Takara Bio Inc., Shiga, Japan), respectively, as described in the product instruction manuals.

Analysis of Interactions of the Se14 Locus with the Ehd1, Ghd7, and Se13 Loci

Four single mutant lines (ehd1, hdl, ghd7 and se13) and four double mutant lines for se14 (se14 ehd1, se14 hdl, se14 ghd7 and se14 se13) were planted under ND conditions in Kyoto in 2012, and examined for flowering time.

Expression Analysis of Flowering Time in HS112

Plants of HS112 were grown in a cabinet with temperature control under a 14.5 h day length (14.5 h light, 30°C/9.5 h dark, 25°C) at 70% relative humidity. Seedlings were grown on sand with additional liquid fertilizer (Kimura’s B Culture Solution, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). On the 30 days before flowering, leaves were collected at 4-hour intervals during that day. Total RNAs were extracted with the Trizol reagent (Life Technologies Inc., Gaithersburg, Maryland, USA) according to the manufacturer’s protocols. Total RNA was subjected to DNA digestion by treatment with RNase-free DNase I (Takara Bio Inc.). The Transcriptor first-strand cDNA synthesis kit (Roche Applied Science, Indianapolis, Indiana, USA)
was used to reverse-transcribe cDNA from 1 μg of RNA using anchored-oligo(dT)18 primers. Real-time PCR analysis was performed by the Taq-Man PCR method using a LightCycler 1.5 (Roche Applied Science) according to the manufacturer’s instructions. The primer sets of Hidl, Ehd1, Hid3a, RFT1, Ghd7, Ehd2 and UBQ genes and Universal Probe Library probes of each gene were designed by ProbeFinder version 2.45 (Roche; https://www.roche-applied-science.com/). Primer and Probe sequences are shown in Table S1. Expression analysis using the standard curve method was performed to determine the expression level of each gene. In order to compare expression levels among the genes, the relative expression level of each gene versus the UBQ expression level was calculated. The RNA gene standards for the seven genes were applied to their plasmids prepared by the pGEM-T Easy Vector System (Promega Corp., Madison, Wisconsin, USA) using PCR amplicons from the total RNA of Gimbozu.

Investigation of H3K4 Methylation State in HS112

Plants of HS112 were grown under a 14.5 h day-length (14.5 h light, 30°C/9.5 h dark, 25°C) at 70% relative humidity conditions. Thirty days after sowing, we collected fully opened leaves from the top of seedlings at two hours after dawn, when the expression of Ehd1, Hid3a and RFT1 were changed in HS112. These leaves were chopped into small fragments and infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking. Chromatin was isolated and sonicated to about 500 bp. Anti-histone H3K4me3 (Abcam Inc., Cambridge, MA, USA) was added to the chromatin solution precleared with magnetic Dynabeads Protein G (Life Technologies Inc.). After subsequent incubation with the Protein G beads, immunocomplexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immunocomplexes were removed by incubation with proteinase K followed by phenol/chloroform extraction. DNA was recovered by ethanol precipitation. The amount of immunoprecipitated chromatin of rice flowering genes was determined by real-time PCR on eight different regions of their respective loci using SYBR Green PCR master mix. The primer sets for the ChIP assay, which are listed in Table S2, were made for every 500 bp within the promoter regions and at 5’UTR, 1st exon and 1st intron of Ehd1, Hid3a and RFT1. The amounts of DNA fragments of each region were normalized to the control samples, which were determined by the ChIP assay with H3 universal antibody.

Supporting Information

Figure S1 Alignment of deduced amino acid sequences of plant ELF6-like proteins. This alignment was generated by the ClustalW routine in the MEGA 5 software (Tamura et al. 2011). The alignment is presented by GENEDOC (www.psc.edu/biomed/genedoc). Residues on black and gray backgrounds indicate 100% and 60% amino acid similarity, respectively. Conserved (black), similar (gray) and non-conserved amino acid residues (white) were highlighted with the GENEDOC software. A dotted line, black line and boxes indicate JmjN, JmjC and ZnF domains, respectively. A triangle indicates the mutation site in Arabidopsis thaliana ELF6: AT3G04240.1; O. sativa ELF6-like gene: Os12g0279100 (LOC_Os12g10150.1); Glycine max GmELF6: XP_005535439; Lotus japonicus L ELF6: Chr5.117T17D03.90.r2.d; Zya mays zmELF6: ZEMMB73_57839. Accession and locus numbers correspond to GenBank IDs.

Figure S2 Diurnal expression of Se14 in WT and the expression of Se14 in the Se13 deficient mutants. Diurnal expression analysis was performed by the standard curve method. For comparing expression levels among the genes, the relative expression level of Se14 against the UBQ expression level was calculated. WT and the Se13 deficient mutant were grown under 14.5 h day-length conditions. Leaves of three plants were sampled at 4 h intervals (three replications) 30 days after sowing. The black bars indicate the dark period, and the white bars indicate the light period.

Table S1 Primer sets for expression analysis.

Table S2 Primer sets for ChIP assay.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TY HS YO. Performed the experiments: TY YY QX TA. Analyzed the data: TY HS YY. Contributed reagents/materials/analysis tools: TY HS YY T. Tsukiyama MT YO T. Tanisaka. Wrote the paper: TY HS YO T. Tsukiyama T. Tanisaka.

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