

# *Hd3a* and *RFT1* are essential for flowering in rice

Reina Komiya, Akiko Ikegami\*, Shojiro Tamaki, Shuji Yokoi† and Ko Shimamoto‡

*RICE FLOWERING LOCUS T 1* (*RFT1/IFT-L3*) is the closest homologue of *Heading date 3a* (*Hd3a*), which is thought to encode a mobile flowering signal and promote floral transition under short-day (SD) conditions. *RFT1* is located only 11.5 kb from *Hd3a* on chromosome 6. Although *RFT1* RNAi plants flowered normally, double *RFT1-Hd3a* RNAi plants did not flower up to 300 days after sowing (DAS), indicating that *Hd3a* and *RFT1* are essential for flowering in rice. *RFT1* expression was very low in wild-type plants, but there was a marked increase in *RFT1* expression by 70 DAS in *Hd3a* RNAi plants, which flowered 90 DAS. H3K9 acetylation around the transcription initiation site of the *RFT1* locus had increased by 70 DAS but not at 35 DAS. In the absence of *Hd3a* and *RFT1* expression, transcription of *OsMADS14* and *OsMADS15*, two rice orthologues of *Arabidopsis* *APETALA1*, was strongly reduced, suggesting that they act downstream of *Hd3a* and *RFT1*. These results indicate that *Hd3a* and *RFT1* act as floral activators under SD conditions, and that *RFT1* expression is partly regulated by chromatin modification.

**KEY WORDS:** *FT-like* gene family, Photoperiodic flowering, Epigenetic regulation, Rice

## INTRODUCTION

The developmental process leading to flowering comprises a vegetative stage and a reproductive stage. The shoot apical meristem (SAM) gives rise to the vegetative structures, eventually transitioning to the reproductive stage that produces flowers. Floral transition is triggered by both endogenous and environmental signals. Among the various environmental signals, photoperiod provides plants with a signal for the most suitable season for flowering (Yanovsky and Kay, 2003; Baurle and Dean, 2006). Plants generally fall into one of three photoperiod-sensing classes: long-day plants (LDP), which promote flowering by sensing long-day (LD) photoperiods; short-day plants (SDP), which promote flowering by sensing short-days (SD); and day-neutral plants, which are not regulated by photoperiod.

The signaling cascades of photoperiodic flowering have been studied in the LDP *Arabidopsis thaliana*. *CONSTANS* (*CO*) encodes a zinc-finger transcriptional activator and induces expression of the floral integrator *FLOWERING LOCUS T* (*FT*) under LD conditions (Kardailsky et al., 1999; Kobayashi et al., 1999; Yanovsky and Kay, 2002). *FT* expression is regulated by both the circadian clock and light (Yanovsky and Kay, 2003; Imaizumi and Kay, 2006). The *CO-FT* pathway is conserved in rice, which is a SDP [*Heading date 1* (*Hd1*)→*Heading date 3a* (*Hd3a*)] (Yano et al., 2000; Hayama et al., 2003). *Hd3a*, which was identified as a quantitative trait locus (QTL) for flowering time, is a key activator of flowering in rice (Kojima et al., 2002). Recent studies suggest that *FT/Hd3a* represents a florigen-type mobile flowering signal (Tamaki et al., 2007; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Lin et al., 2007). *Hd3a* expression is regulated by *Hd1*, and by *Ehd1*, a B-type response regulator that functions independently of *Hd1* (Yano et al., 2000; Hayama et al., 2003; Doi et al., 2004). *Hd3a* is also regulated by light via the phytochrome B sensory system.

These two functional pathways merge at *Hd3a* (Izawa et al., 2002; Ishikawa et al., 2005). Key regulators for photoperiodic flowering in rice and *Arabidopsis* are conserved, but differences in their regulation result in either SDP or LDP (Hayama et al., 2003).

In addition to the photoperiodic pathway, vernalization, autonomous and gibberellin pathways are integrated into the transcriptional regulation of downstream target genes such as *FT*, *TWIN SISTER OF FT* (*TSF*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) in *Arabidopsis* (Boss et al., 2004; Imaizumi and Kay, 2006). Prolonged exposure to cold, a process known as vernalization, promotes flowering in winter annual *Arabidopsis*. *FLOWERING LOCUS C* (*FLC*), a MADS-box transcription factor, suppresses floral transition by repressing the expression of floral activators (Michaels and Amasino, 1999). *FLC* expression is repressed by the vernalization pathway through epigenetic mechanisms at the *FLC* locus (Sung and Amasino, 2004a; He and Amasino, 2005). Vernalization requires *VERNALIZATION INSENSITIVE 3* (*VIN3*), a member of a plant-specific protein family with plant homeodomain and fibronectin domains, *VERNALIZATION 2* (*VRN2*), a homologue of polycomb group protein, and *VERNALIZATION 1* (*VRN1*), a protein containing a DNA-binding domain (Levy et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004b). These genes are involved in H3K9-mediated deacetylation, and H3K9- and H3K27-mediated dimethylation chromatin modifications at the first intron of *FLC*, and promote flowering by the suppression of *FLC*. Furthermore, *HETEROCHROMATIN PROTEIN 1* (*LHP1*)/*TERMINAL FLOWER II* (*TFLII*) is required to maintain the increased level of H3K9 dimethylation at the *FLC* locus (Sung et al., 2006). In rapid-cycling accessions of *Arabidopsis*, *FLC* expression is also regulated by the autonomous pathway, which constitutively represses flowering. In this pathway, *FLOWERING LOCUS D* (*FLD*) and *FVE*, plant homologues of a protein found in the histone deacetylase (HDAC) complex of mammals, partly regulate flowering by histone deacetylation at the *FLC* locus (He et al., 2003; Ausin et al., 2004). Chromatin modifications at the *SOC1* locus have also been observed (Bouvet et al., 2006). However, chromatin modifications at the *FT* locus have not been reported (Sung et al., 2006), although *FT* expression is regulated by *FLC*. In *Arabidopsis*, flowering is regulated by many floral activators through multiple pathways, but there is no *FLC* orthologue in the

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rice genome (Goff et al., 2002; Doi et al., 2004), and rice does not require vernalization for flowering. Photoperiodic flowering is thus the key pathway in rice, but no report on floral regulation through chromatin modification has as yet been published.

In *Arabidopsis*, *TSF*, an *FT* homologue, acts redundantly with *FT* to promote floral transition, because *ft tsf* double mutants flower slightly later than *ft* single mutants (Michaels et al., 2005; Yamaguchi et al., 2005). The rice genome contains thirteen members of the *Hd3a* gene family (Chardon and Damerval, 2005). *RFT1/FT-L3* is the closest homologue of *Hd3a*, and *FTL/FT-L1* is the second closest homologue. Transgenic rice plants overexpressing *RFT1* or *FTL* flower early, much like *Hd3a*-overexpressing plants (Izawa et al., 2002; Kojima et al., 2002). However, because there are no mutants of *FT-like* genes available, including *Hd3a*, it is unclear whether *FT-like* genes other than *Hd3a* function as floral activators. In this study, we show that double *RFT1-Hd3a* RNAi plants do not flower for up to 300 days after sowing (DAS), indicating that these two genes are essential for flowering in rice. Moreover, *RFT1* functions as a floral activator in *Hd3a* RNAi plants. *OsMADS14* and *OsMADS15* were shown to be downstream of *Hd3a* and *RFT1* in rice flowering under SD conditions. On the basis of these results, we propose a model for the regulation of rice flowering under SD conditions.

## MATERIALS AND METHODS

### Plant materials and growth conditions

*Japonica* rice cultivar Norin 8 was used as wild type in the expression, DNA methylation and ChIP assays. *Japonica* rice cultivar Nipponbare was used as wild type in the expression analysis of the *hd1* mutant. The *Tos17*-induced mutant of *Hd1* was described previously (Ishikawa et al., 2005). Plants were grown in climate chambers at 70% humidity under SD conditions with daily cycles of 10 hours of light at 30°C and 14 hours of dark at 25°C, or LD conditions with 14 hours of light and 10 hours of dark. Light was provided by fluorescent white light tubes (400 to 700 nm, 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

### *Hd3a* RNAi, *RFT1* RNAi, double *RFT1-Hd3a* RNAi and *RFT1::GUS* constructs

To generate RNAi transgenic plants, the gene sequences of *Hd3a* and *RFT1*, for which inverted repeats were made, were amplified using specific primers (see Table 1) and subcloned into the pENTR/D-TOPO cloning vector (Invitrogen) to yield entry vectors. The final RNA silencing vectors were produced by an LR clonase reaction between each of the entry vectors and pANDA (Miki and Shimamoto, 2004). A 1.7 kb promoter region of the *RFT1* gene was used to construct *RFT1::GUS*, including an intron to enhance *GUS* expression (Tanaka et al., 1990). Transgenic rice plants were generated by *Agrobacterium*-mediated transformation of rice calli (cv. Norin 8), performed according to a published protocol (Hiei et al., 1994).

### Histochemical analysis of GUS expression

GUS staining was described previously (Moritoh et al., 2005). Samples were embedded in paraffin and sectioned at a thickness of 10  $\mu\text{m}$  using an ULTRACUT UCT ultramicrotome (Leica). Sections were photographed using a BX50 microscope (Olympus).

### RNA extraction and real-time PCR analysis

Leaves were harvested at various times of the day, and total RNA was extracted using an RNeasy plant mini kit (Qiagen) and treated with DNase I (Invitrogen). cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using SuperScriptII Reverse Transcriptase (Invitrogen). One microlitre of cDNA was used for the quantitative analysis of gene expression performed with SYBR Green PCR master mix (Applied Biosystems) with gene-specific primers (see Table 1). Data were collected using the ABI PRISM 7000 sequence detection system in accordance with the instruction manual.

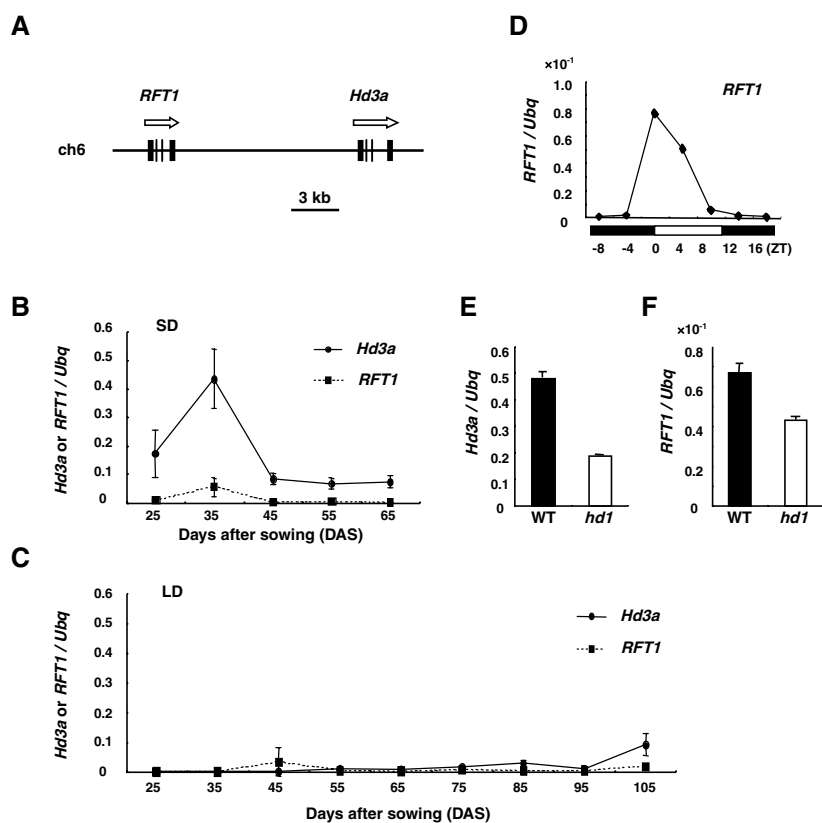
### Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed as described previously (Nagaki et al., 2003; Nagaki et al., 2004; Okano et al., 2008) using whole leaves harvested 35 or 70 DAS (zeitgeber time; ZT 0) under SD conditions from *Hd3a* RNAi and

**Table 1. Primer sequences used for expression, DNA methylation and ChIP analysis**

Primer name	Sequence (5' to 3')
<b>Primers for expression analysis</b>	
<i>Ubq</i> -F	AACCAGCTGAGGCCAAGA
<i>Ubq</i> -R	ACGATTGATTAACCAGTCCATGA
<i>Hd3a</i> -F	GCTCACTATCATCATCCAGCATG
<i>Hd3a</i> -R	CCTTGCTCAGCTATTTAATTGCATAA
<i>RFT1</i> -F	TGGTTAGCTGACCTAGATTCAAA
<i>RFT1</i> -R	GCCAACCACAAGAGGATCGT
<b>Primers for RNAi constructs</b>	
<i>Hd3a</i> -F	CACCTGCTGCATGCTCACTATCATC
<i>Hd3a</i> -R	CATGAGAGACCTTAGCCTTGC
<i>RFT1</i> -F	CACCGGCTAGCTTAACCTTCTGAACATC
<i>RFT1</i> -R	GCCGGCCATGTCAAATTAATAACC
<b>Primers for <i>RFT1::GUS</i></b>	
<i>RFT1</i> promoter-F	TGATATTCTCGCACCCAGTCTTG
<i>RFT1</i> promoter-R	CCCACTAACTTATGAAGCTAGCCGG
<b>Primers for screening RNAi plants</b>	
<i>Hd3a</i> -F	GTCTACTTCAACTGCCAGCGCGAG
<i>Hd3a</i> -R	GAACCTGCAATGTATAGCATGCTGG
<i>RFT1</i> -F	GTCTACTTCAACTGCCAGCGCGAG
<i>RFT1</i> -R	CTTAGCTATAGCTGCTGCATGCATG
<b>Primers for <i>OsMADS</i>s expression analysis</b>	
<i>OsMADS14</i> -F	CGGTTGCGAGACGAGGAA
<i>OsMADS14</i> -R	GAAAGACGGTGCTGGACGAA
<i>OsMADS15</i> -F	CGTCGTCGGCCAAACAG
<i>OsMADS15</i> -R	TGACTTCAATTCATTCAAGTTGCT
<i>OsMADS50</i> -F	CAGGCCAGGAATAAGCTGGAT
<i>OsMADS50</i> -R	TTAGGATGGTTGGTGTCTATTGC
<b>Primers for ChIP analysis</b>	
<i>Act</i> -F	ATGGGGCTCTCGGATGTAG
<i>Act</i> -R	TAGTGCCCTTTTCCCCTCTT
I-F	CAGTTTCAGCACTTCTATCGACATG
I-R	AGCCTTACTATGATGTGCCAGTAG
II-F	TAGTGTCTTGGGTTGGTACTGCCTA
II-R	CCGTTCAATCCAGAGGATAGATGTC
III-F	CAGGAGATACCTAAGCTAGCTAGCA
III-R	AACCCACTAATTTATGAAGCTAGCC
IV-F	GGATGCAGAGACCCACCAGAAGTTA
IV-R	GGGTCTACCATCACCTGTAGGTAAT
V-F	GTAGGCACCGATCAGATATGTTAGC
V-R	CTCCAGTGGTACCAGGAATATCGGT
VI-F	CGTCAGATTTGAAGGATAGGGCTGT
VI-R	ACATCACCTCTTGCCTGCATCAAT
VII-F	GTCTACTTCAACTGCCAGCGCGAG
VII-R	CTTAGCTATAGCTGCTGCATGCATG
<b>Primers for DNA methylation analysis</b>	
35S M-F	AAACCTCCTCGATTCCATT
35S M-R	AGCCTGTCTCTCCAATGA
<i>RFT1</i> I M-F	GCCATGCATCCATCAAGAAAATCC
<i>RFT1</i> I M-R	CAAAGCATGCATGCATATGTGGAGG
<i>RFT1</i> II M-F	CCTCCACATATGCATGCATGCTTTG
<i>RFT1</i> II M-R	CGTTCAATCCAGAGGATAGATGTC
<i>RFT1</i> III M-F	GACATCTATCCTCTGGATTGAACG
<i>RFT1</i> III M-R	ACTCCCTAAGGTTAGGGTTGCTTGG

wild-type plants. Isolated nuclei were digested with micrococcal nuclease (Sigma) instead of sonication and, after the recovery of nucleosomes, we confirmed that monomer nucleosome (~160 bp) was most abundant by electrophoresis. ChIP-PCR products were quantified by real-time PCR. Quantitative ChIP-PCR was normalized to *Actin1* in each experiment.



**Fig. 1. Expression of *RFT1* is similar to *Hd3a* under SD and LD conditions.** (A) Schematic diagram of *RFT1* and *Hd3a* on chromosome 6. The physical distance between *RFT1* and *Hd3a* is 11.5 kb. (B,C) Developmental expression analysis of *RFT1* and *Hd3a* under SD (B) and LD (C) conditions. Expression of both *RFT1* and *Hd3a* increased by 35 days after sowing (DAS), when reproductive transition occurred under SD conditions (B), but not under LD conditions (C). Leaves were collected from wild-type plants (ZT 0; zeitgeber time). (D) Diurnal expression of *RFT1* 35 DAS under SD conditions. *RFT1* and *Hd3a* show diurnal expression patterns with a peak at ZT 0 (ZT 0=light on). (E,F) Expression analysis of *RFT1* and *Hd3a* in *hd1* mutants under SD conditions. *Hd1* mutant was induced by *Tos17*, which was inserted in exon 1 of *Hd1*. Leaves were collected from wild-type or *hd1* plants 35 DAS (ZT 0) under SD conditions.

Regions I-VII of the *RFT1* locus were amplified by real-time PCR using specific primers (see Table 1). ChIP assays were performed three times with at least two replicates each for each sample.

#### DNA methylation assay

Genomic DNA from plants grown under SD conditions was extracted at 35 and 70 DAS from leaves of wild-type and *Hd3a* RNAi plants using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). Genomic DNA (1  $\mu$ g) was digested with McrBC as recommended by the supplier (New England BioLabs). McrBC is an endonuclease, which cleaves DNA containing methylcytosine. Digested DNA template (1  $\mu$ l) was then amplified, using PCR, for 25 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 60 seconds at 72°C with gene-specific primers (see Table 1). M65, which is a transgenic line of cv. Nipponbare that carries non-methylated 35S-*GFP* (Hashizume et al., 1999), and the 35S-Pi line, which carries a silencer construct and methylated 35S-*GFP* (Okano et al., 2008), served as controls. Methylation status of 35S-*GFP* was determined by bisulfite sequencing (Okano et al., 2008).

## RESULTS

### Expression of *RFT1* is similar to *Hd3a* under SD and LD conditions

In rice, *RFT1* is the closest homologue of *Hd3a*, with 91% identity in the deduced amino acid sequence (Kojima et al., 2002; Chardon and Damerval, 2005; Faure et al., 2007). *RFT1* also lies adjacent to *Hd3a*, separated by only 11.5 kb on chromosome 6 (Fig. 1A). To examine *RFT1* expression during different developmental stages under SD and LD conditions, we sampled leaves from wild-type plants every 10 days from 25 DAS until flowering. Levels of *Hd3a* and *RFT1* mRNA were examined by quantitative RT-PCR. Under SD conditions, levels of *RFT1* and *Hd3a* transcripts were highest 30 days before flowering, concurrent with floral transition, although absolute transcript levels of *RFT1* were much lower than those of

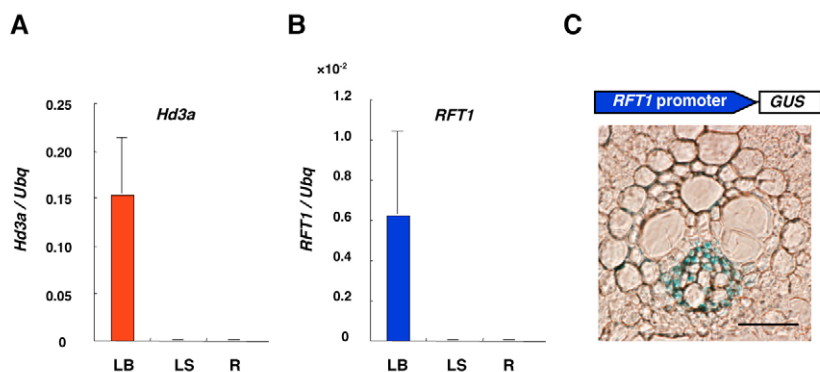
*Hd3a* (Fig. 1B). Expression of no other *FT-like* gene peaked 30 days before flowering (data not shown). *Hd3a* expression is diurnal with a peak before dawn, and a gradual decrease during the day under SD conditions (Izawa et al., 2002). Our analysis showed that *RFT1* expression is also diurnal, with a peak before dawn (Fig. 1D). It is known that *Hd3a* acts downstream of the photoperiod pathway via *Hd1* transcriptional regulation (Kojima et al., 2002; Hayama et al., 2003). Expression of *RFT1* in *hd1* mutants was significantly reduced under SD conditions, as is *Hd3a* (Fig. 1E,F), indicating that *RFT1* and *Hd3a* are partially regulated by *Hd1* under SD conditions. Under LD conditions, expression of *Hd3a* and *RFT1* was barely detectable in any developmental stage in wild-type plants (Fig. 1C).

To study the spatial pattern of *RFT1* expression, *RFT1* and *Hd3a* expression was measured in leaf blades, sheaths and roots under SD conditions. Expression of *RFT1* and *Hd3a* was observed in leaf blades, but not in leaf sheaths or roots (Fig. 2A,B). An *RFT1::GUS* reporter fusion protein was detected in leaf blade vascular tissues 35 DAS under SD conditions (Fig. 2C). This expression pattern is similar to that of *Hd3a::GUS* (Tamaki et al., 2007). The similarity of *Hd3a* and *RFT1* expression patterns under SD and LD conditions, and in vascular tissues, suggests that *RFT1* could function redundantly with *Hd3a* in promoting floral transition under SD conditions.

### *Hd3a* and *RFT1* are essential for flowering in rice under SD conditions

To test whether *RFT1* affects floral induction under SD conditions, we produced transgenic plants that suppress *RFT1*, *Hd3a*, or both (Fig. 3D). Because homology between *RFT1* and *Hd3a* is low in the 5' and 3' non-coding regions, the 5'UTR of *RFT1* was used for the RNAi construct to specifically suppress *RFT1* expression and the 3'UTR of *Hd3a* was used to specifically suppress *Hd3a* expression (Fig. 3A,B). The flowering time of *RFT1* RNAi plants ( $T_1$ ) was





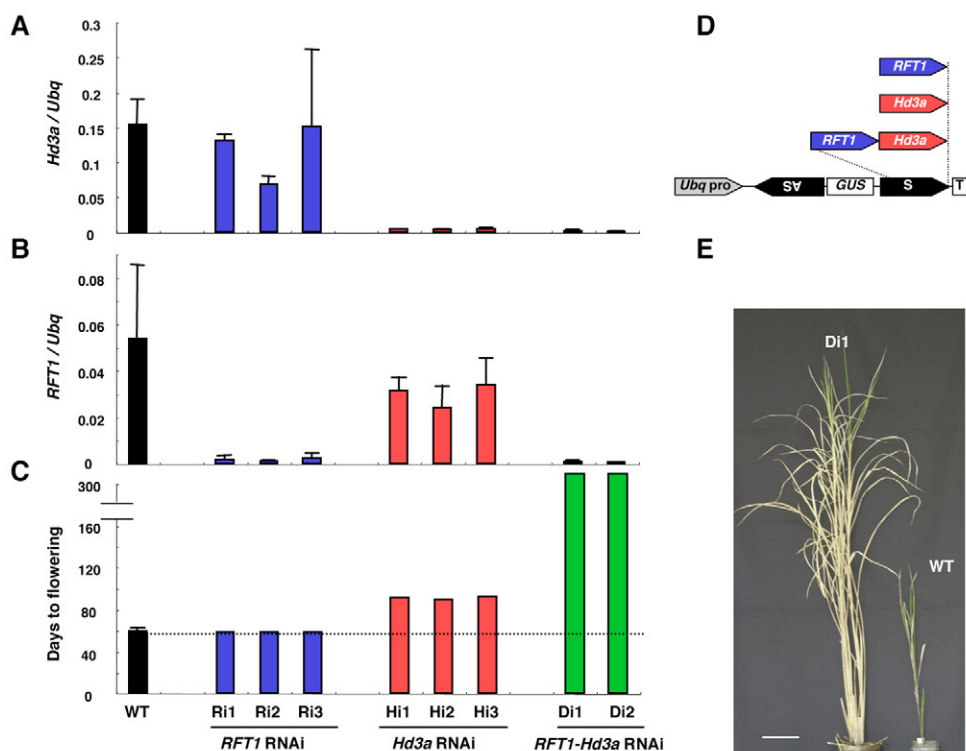
**Fig. 2. *RFT1* expression is localized in leaf vascular tissues.** (A,B) Quantitative RT-PCR analysis of *Hd3a* and *RFT1* expression in rice tissues under SD conditions. *RFT1* and *Hd3a* were expressed in leaf blades (LB), but not in leaf sheaths (LS) or roots (R). Leaf blade, leaf sheaths and roots were collected from wild-type plants grown under SD conditions 35 DAS (ZT 0). (C) *RFT1* promoter activity in *RFT1::GUS* transgenic plants. Transverse leaf section in *RFT1::GUS* plants 35 DAS under SD conditions (ZT 4). Scale bars: 20  $\mu$ m.

essentially the same as in wild type ( $59 \pm 3.5$  DAS,  $n=9$  for wild type versus  $62 \pm 8.3$  DAS,  $n=18$  for *RFT1* RNAi plants) under SD conditions (Fig. 3C). This result indicates that *Hd3a* acts as the primary activator of flowering in *RFT1* RNAi plants, and that *RFT1* does not contribute significantly to floral transition under SD conditions. By contrast, *Hd3a* RNAi plants ( $T_1$ ) flowered  $95 \pm 6.4$  DAS ( $n=9$ ) under SD conditions, about 30 days later than did wild type (Fig. 3C). New leaves are not normally produced after floral transition in rice. Until 60 DAS, *Hd3a* RNAi and wild-type plants had the same number of leaves, indicating that the growth rates of *Hd3a* RNAi and wild-type plants are about the same (data not shown). However, *Hd3a* RNAi plants produced two or three more leaves than did wild-type plants after 60 DAS, suggesting that floral transition is delayed in *Hd3a* RNAi plants. It is likely, then, that *Hd3a*, but not *RFT1*, promotes floral transition under SD conditions. Double *RFT1-Hd3a* RNAi plants did not flower up to 300 DAS ( $n=10$ ) under SD conditions (Fig. 3C). These plants continued to produce leaves for 300 days and reached a height of 110-130 cm, about double the height of wild-type plants (Fig. 3E). The absence

or extended delay of flowering in double *RFT1-Hd3a* RNAi plants is apparently due to a complete defect in floral transition. These results suggest that *Hd3a* and *RFT1* are essential for flowering in rice.

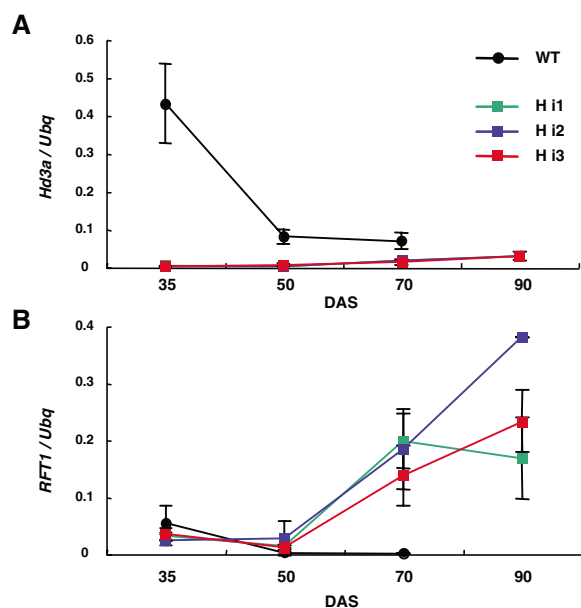
### *RFT1* expression is much higher in *Hd3a* RNAi plants at later developmental stages

The 30-day delay in flowering of *Hd3a* RNAi plants suggests that additional activators trigger flowering in *Hd3a* RNAi plants. The observation that double *RFT1-Hd3a* RNAi plants did not flower even 300 DAS lends credence to the possibility that *RFT1* acts as a floral activator in *Hd3a* RNAi plants. Transcript levels of *RFT1* were very low in wild-type plants throughout development, but, in *Hd3a* RNAi plants, expression gradually increased from 50 DAS to 70 DAS, which was about 30 days before flowering, and is consistent with the timing of floral transition. *RFT1* expression continued to increase until flowering at 90 DAS (Fig. 4). Because *RFT1* RNAi had no effect on the timing of floral transition (Fig. 3B,C), *RFT1* is not likely to play a role in flower induction under SD conditions in



**Fig. 3. Functional analysis of *RFT1* by RNAi under SD conditions.**

(A) *Hd3a* expression in *RFT1* RNAi, *Hd3a* RNAi and double *RFT1-Hd3a* RNAi plants under SD conditions. (B) *RFT1* expression in RNAi plants under SD conditions. Leaves were sampled 35 DAS (ZT 0) under SD conditions. Expression of *Hd3a* and *RFT1* was examined by real-time PCR. Black, wild-type; blue, *RFT1* RNAi plants; red, *Hd3a* RNAi plants; green, double *RFT1-Hd3a* RNAi plants. (C) Flowering time of RNAi plants under SD conditions. (D) RNAi constructs. Gene-specific regions of *Hd3a* and *RFT1* were used for the *Hd3a* and *RFT1* RNAi constructs. 5' UTR of *RFT1* and 3' UTR of *Hd3a* were used for the double *RFT1-Hd3a* constructs. (E) Growth of wild-type and *RFT1-Hd3a* RNAi plants. Ri 1-3, *RFT1* RNAi plants; Hi 1-3, *Hd3a* RNAi plants; Di 1-2, double *RFT1-Hd3a* RNAi plants. Scale bars: 10 cm.

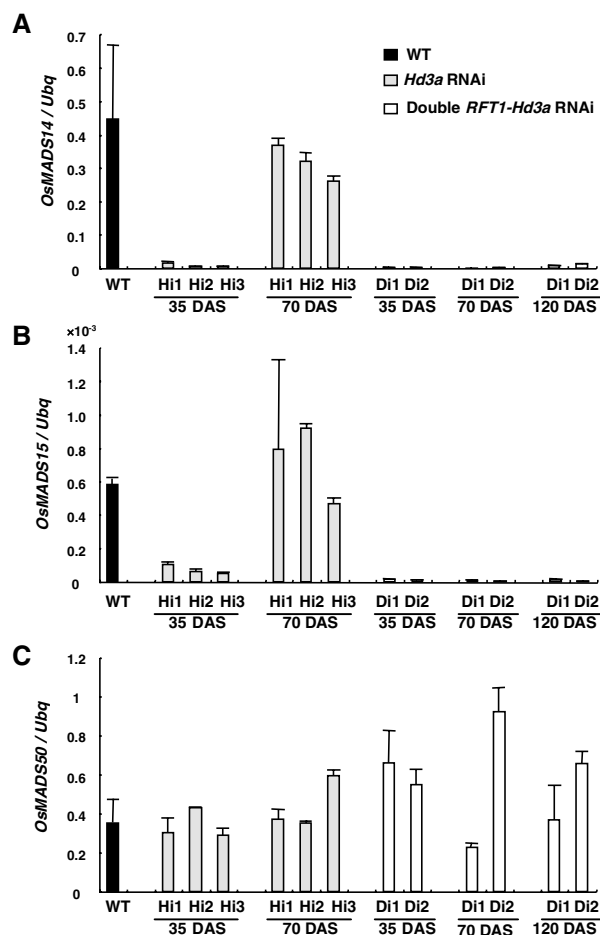


**Fig. 4. Expression of *RFT1* in *Hd3a* RNAi plants under SD conditions.** (A) Expression of *Hd3a* in *Hd3a* RNAi and wild-type plants. *Hd3a* expression was suppressed at all developmental stages in *Hd3a* RNAi plants. (B) Expression of *RFT1* in *Hd3a* RNAi and wild-type plants. *RFT1* transcription was very low in wild-type plants (black line). However, in *Hd3a* RNAi plants, *RFT1* expression (green, blue and red lines) had increased 30 days before flowering, the stage required for promoting reproductive transition. Leaves were collected from wild-type plants and *Hd3a* RNAi plants at 35, 50, 70 and 90 DAS (ZT 0) under SD conditions.

wild-type plants. However, in the absence of *Hd3a* expression, as in *Hd3a* RNAi plants, the marked increase of *RFT1* expression at a later stage is highly correlated with the induction of flowering. Expression of *RFT1* in *Hd3a* RNAi plants was more than 50-fold higher than in wild-type plants. However, no other *FT*-like genes had significantly increased expression. Because increased *RFT1* expression was also found in other RNAi plants, in which *Hd3a* expression was suppressed by constructs containing the coding region of *Hd3a*, this phenomenon is likely to be caused by the absence of *Hd3a* expression, not by sequences used for the RNAi construct (data not shown). The increase in *RFT1* expression was observed only when plants were grown under inductive SD conditions. Under LD conditions, when *Hd3a* expression was low, no increase was observed in *RFT1* expression. Together, these results suggest that *RFT1* functions as a floral activator in the absence of *Hd3a* expression under SD conditions.

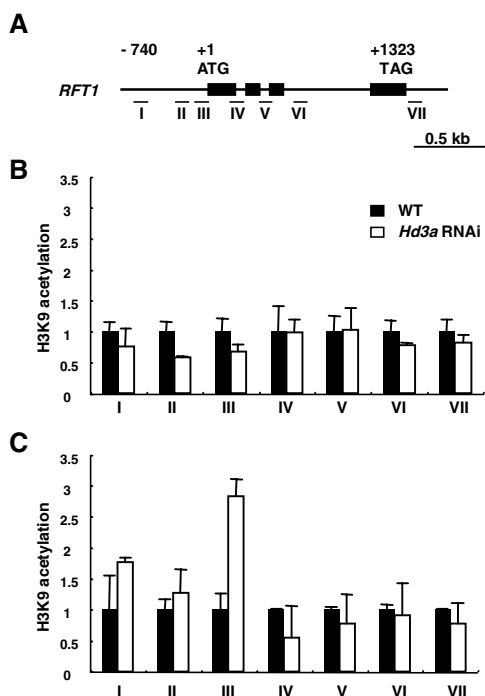
### ***Hd3a* and *RFT1* act upstream of *OsMADS14* and *OsMADS15***

In *Arabidopsis*, *APETALA1* (*API*), *SEPALLATA3* (*SEP3*), *FRUITFULL* (*FUL*) and *SOC1* are induced by *FT* in leaves and/or the SAM (Abe et al., 2005; Michaels et al., 2005; Teper-Bammler and Samach, 2005). In rice, *OsMADS1*, *OsMADS14* and *OsMADS15* are upregulated in the floral meristem when it begins to differentiate into primary panicle branch primordia (Furutani et al., 2006). To identify genes acting downstream of *Hd3a* and *RFT1*, we examined the expression of *OsMADS14*, *OsMADS15* and *OsMADS50* at 35 and 70 DAS in leaves of *Hd3a* RNAi and double



**Fig. 5. Expression of *OsMADS14*, *OsMADS15* and *OsMADS50* in *Hd3a* RNAi and double *RFT1*-*Hd3a* RNAi plants.** (A-C) Expression of *OsMADS* genes 35 and 70 DAS in *Hd3a* RNAi and double *RFT1*-*Hd3a* RNAi plants under SD conditions. Leaves were collected from *Hd3a* RNAi plants at 35 and 70 DAS, or double *RFT1*-*Hd3a* RNAi plants at 35, 70 and 120 DAS (ZT 0), under SD conditions. Expression of *OsMADS14* (A) and *OsMADS15* (B), rice orthologues of *Arabidopsis* *API*, was suppressed at 35 DAS, but increased at 70 DAS, when *RFT1* expression was increased in *Hd3a* RNAi plants. In double *RFT1*-*Hd3a* RNAi plants, expression of *OsMADS14* (A) and *OsMADS15* (B) was suppressed at 35, 70 and 120 DAS. Expression of *OsMADS50* (C), a *SOC1* orthologue, was not suppressed in *Hd3a* RNAi and double *RFT1*-*Hd3a* RNAi plants. Hi 1-3, *Hd3a* RNAi plants; Di 1-2, double *RFT1*-*Hd3a* RNAi plants.

*RFT1*-*Hd3a* RNAi plants under SD conditions. *OsMADS14* and *OsMADS15* are orthologues of *API*, and *OsMADS50* is a rice orthologue of *SOC1* (Jeon et al., 2000; Lee et al., 2004). *OsMADS14* and *OsMADS15* were suppressed in *Hd3a* RNAi plants 35 DAS (Fig. 5A,B). *OsMADS14* and *OsMADS15* thus apparently act downstream of *Hd3a*. Expression of *OsMADS14* and *OsMADS15* was higher at 70 DAS, when *RFT1* expression was also higher in *Hd3a* RNAi plants (Fig. 5A,B). Furthermore, *OsMADS14* and *OsMADS15* were suppressed at all stages (35, 70 and 120 DAS) in double *RFT1*-*Hd3a* RNAi plants (Fig. 5A,B), suggesting that *RFT1* activates the expression of *OsMADS14* and *OsMADS15*. The expression of *OsMADS50* in *Hd3a* RNAi and double *RFT1*-*Hd3a* RNAi plants was similar to that in wild-type plants (Fig. 5C). These



**Fig. 6. Histone modifications at the *RFT1* locus in *Hd3a* RNAi plants.** (A) Schematic diagram of *RFT1*. I through VII represent the regions in which H3K9 acetylation was examined by ChIP. The translation initiation site is +1. Filled boxes represent exons. (B,C) ChIP analysis of H3K9 acetylation levels of *RFT1*. Relative levels of H3K9 acetylation at various regions of the *RFT1* locus were assayed at 35 DAS (B) and 70 DAS (C) in *Hd3a* RNAi and wild-type plants under SD conditions. Black, wild-type plants; white, *Hd3a* RNAi plants.

results show that, under SD conditions, *Hd3a* and *RFT1* promote floral transition and induce the expression of *OsMADS14* and *OsMADS15*, but not of *OsMADS50*.

#### ***RFT1* may be regulated by chromatin modification**

The increase in *RFT1* expression in *Hd3a* RNAi plants had occurred by 70 DAS, which is 35 days later than the normal peak observed in wild-type plants, indicating that activation of flowering by *RFT1* is slower than by *Hd3a* (Fig. 4). Winter-annual accessions of *Arabidopsis* promote flowering by vernalization. The vernalization process has evolved to distinguish long exposures to cold from shorter exposures, a safeguard that prevents flowering during short-term autumnal temperature fluctuations. *FLC* expression is epigenetically suppressed by a number of chromatin-remodeling factors. The gradual activation of *RFT1* expression in *Hd3a* RNAi plants might thus be the result of epigenetic regulation.

We first assessed DNA methylation at the *RFT1* locus at 35 and 70 DAS, because *RFT1* expression was very low at 35 DAS and very high at 70 DAS in *Hd3a* RNAi plants. After digestion of genomic DNA with MspBC, an endonuclease that cleaves DNA containing methylcytosine, specific primers were used to amplify the MI, MII and MIII regions of the *RFT1* locus (see Table 1). DNA methylation was not detected in any region of the *RFT1* locus at any stage in either wild-type or *Hd3a* RNAi plants (see Fig. S1 in the supplementary material). We obtained similar results when DNA methylation at the *RFT1* locus was analyzed by PCR after treatment with methylation-sensitive restriction enzymes (data not shown).

DNA methylation was not altered 35 or 70 DAS in wild-type plants or *Hd3a* RNAi plants, suggesting that the increased expression of *RFT1* in *Hd3a* RNAi plants was not associated with DNA methylation.

Chromatin immunoprecipitation (ChIP) assays were used to examine histone modifications at the *RFT1* locus. H3K9 or H4 acetylation and H3K4 dimethylation cause major modifications of active chromatin, whereas H3K9 dimethylation and H3K27 dimethylation are heterochromatic markers (Fuchs et al., 2006). Chromatin modifications in regions I through VII of the *RFT1* locus in *Hd3a* RNAi plants were compared with those in wild-type plants at 35 and 70 DAS by ChIP, using antibodies against H3K9 acetylation (Fig. 6A). At 70 DAS, when *RFT1* expression is highly activated in *Hd3a* RNAi plants, levels of H3K9 acetylation were higher than wild-type plants in region III, the region around the start site of transcription (Fig. 6C). By contrast, there was no increase in H3K9 acetylation 35 DAS in *Hd3a* RNAi plants, a stage at which *RFT1* expression is low (Fig. 6B). Increased H3K9 acetylation at the *RFT1* locus may thus be correlated with the activation of *RFT1* transcription.

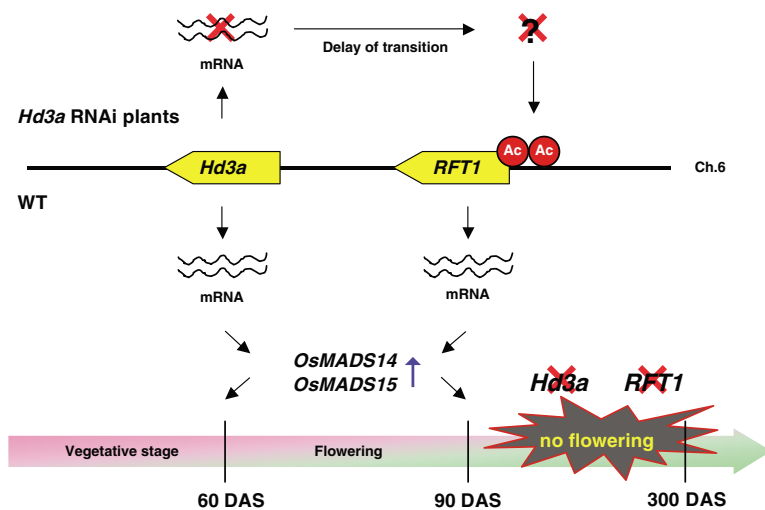
## **DISCUSSION**

### ***RFT1* is a unique member of the *FT-like* gene family in rice**

*FT-like* genes are present in *Arabidopsis*, *Populus*, *Picea*, tomato and barley (Bohlenius et al., 2006; Lifschitz et al., 2006; Faure et al., 2007; Gyllenstrand et al., 2007). Double mutants of *TSF*, a close *Arabidopsis* homologue of *FT*, and *FT* flower late, but they do eventually flower (Michaels et al., 2005; Yamaguchi et al., 2005).

There are 13 rice genes in the *FT-like* gene family (Chardon and Damerval, 2005; Faure et al., 2007). In double *RFT1-Hd3a* RNAi plants, expression of *FT-L4*, *FT-L5*, *FT-L6* and *FT-L12* was similar to that of wild-type plants at 35 DAS and later stages. Expression of *FT-L7*, *FT-L8*, *FT-L9*, *FT-L10*, *FT-L11* and *FT-L13* was barely detectable in wild-type plants. Interestingly, expression of *FT-L1/FTL*, which was the second closest homologue of *Hd3a* in rice, was suppressed in the leaves of double *RFT1-Hd3a* RNAi plants (data not shown). In the shoot apex of wild-type plants, expression of *FT-L1/FTL* was not increased during the transition to the reproductive stage, but was later increased during spikelet and floral organ initiation in the inflorescence meristem (R.K. and K.S., unpublished). Expression of *RFT1* and *Hd3a* was not detected at any stage in the shoot apex (R.K. and K.S., unpublished). Furthermore, in *Hd3a* RNAi plants, expression of *FT-L1/FTL* was suppressed at 35 DAS, and not increased at 70 DAS. These results indicate that *RFT1* is the only member of rice *FT-like* gene family that was upregulated in *Hd3a* RNAi plants (Fig. 4). However, the possibility that *FT-L1/FTL* is involved in the extremely late flowering of the double *RFT1-Hd3a* RNAi plants cannot be completely excluded.

Phylogenetic analysis of cereal *FT-like* genes indicates that *RFT1* is unique to the rice genome, although other *FT-like* genes are found in other cereals (Chardon and Damerval, 2005). *RFT1* and *Hd3a* are physically very close on chromosome 6, separated by only 11.5 kb (Fig. 1A), suggesting that *RFT1* may have arisen by tandem duplication of *Hd3a* after the divergence of rice from some progenitor cereal. Therefore, *RFT1* may function as an auxiliary to *Hd3a* in the flowering developmental pathway when *Hd3a* is suppressed. Regulation by two members of the *FT/Hd3a* gene family involved in flowering may be a rice-specific mechanism, or an as yet undiscovered auxiliary mechanism in other plants.



**Fig. 7. A model for photoperiodic flowering in rice.** In wild-type plants, *Hd3a* promotes transition to the reproductive stage and induces the expression of *OsMADS14* and *OsMADS15*, and flowers about 60 DAS under SD conditions. However, in *Hd3a* RNAi plants, *RFT1* expression starts to increase during later stages, and induces *OsMADS14* and *OsMADS15* expression under SD conditions. When *RFT1* expression is activated, H3K9 acetylation levels are high at the 5' UTR of *RFT1*. Double *RFT1*-*Hd3a* RNAi plants did not flower up to 300 DAS under SD conditions, suggesting that *RFT1* and *Hd3a* are the key regulators of photoperiodic flowering under SD conditions.

### Molecular mechanism model for *RFT1* activation in *Hd3a* RNAi plants

Two mechanisms were initially considered to explain the activation of *RFT1* in *Hd3a* RNAi plants: one was the direct interaction of *Hd3a* mRNA or protein with *RFT1* mRNA; and the other was a de novo adaptive pathway, which arises due to the extended vegetative stage caused by a lack of *Hd3a*. Direct suppression by *Hd3a* mRNA or protein was ruled out by co-transfection assays. *RFT1::GUS* and *Ubq::bar::GFP* (control) or *Ubq::Hd3a::GFP* were co-transfected into rice protoplasts and the effect of *RFT1::GUS* fusion protein activity on *Ubq::Hd3a::GFP* was measured. The absence of *RFT1::GUS* suppression suggested that neither *Hd3a* mRNA nor *Hd3a* protein acts directly on the *RFT1* promoter (data not shown).

The possibility that a short phase of vegetative growth and transition to reproductive stage prevent *RFT1* expression was also considered. Under LD conditions, wild-type expression of *RFT1* does not increase during late stages, when *Hd3a* expression is very low and the vegetative stage is longer than under SD conditions (Fig. 1C). This suggests that delay of the phase change is not the sole cause for the increase of *RFT1* expression. In variety Taichung 65, which carries loss-of-function *Ehd1* and *Hd1* alleles (Doi et al., 2004), expression of *RFT1* is not increased at 70 DAS under SD conditions (see Fig. S2 in the supplementary material). These results suggest that activation of *RFT1* requires *Hd1* and *Ehd1*, and a novel adaptive pathway induced by the loss of *Hd3a* expression (Fig. 7).

To test the possible involvement of epigenetic phenomena in this pathway, we examined histone modifications at the *RFT1* locus by ChIP assays and found that H3K9 acetylation increased in the region around the transcription start site of *RFT1* when *RFT1* was highly expressed in *Hd3a* RNAi plants (Fig. 6C). This suggests that some chromatin-associated factor(s) regulates *RFT1* transcription.

*Ehd1* has a GARP [*Golden2*, *Arabidopsis* *RESPONSE REGULATOR* (*ARR*), and *Chlamydomonas* regulatory protein of P-starvation acclimatization response (*Psr1*)] DNA-binding motif, which specifically recognizes 5-bp oligonucleotides in vitro (Sakai et al., 2000). Binding sites of *Ehd1* are present in *RFT1* promoter region II, in which H3K9 acetylation was slightly increased. H3K9 acetylation of region III (5' UTR), which is adjacent to region II, was highly increased in *Hd3a* RNAi plants. Therefore, chromatin modification at region III of the *RFT1* locus may allow *Ehd1* to bind to the promoter region of *RFT1* and thus induce transcription.

Because region III of the *RFT1* locus has no homology with the *Hd3a* 5' UTR used for the *Hd3a* RNAi construct, siRNA derived from the *Hd3a* RNA constructs was not likely to be involved in chromatin modification of the *RFT1* locus in *Hd3a* RNAi plants. *RFT1* expression also increased in plants in which *Hd3a* expression was decreased by a construct using the coding region of *Hd3a* (data not shown). These results suggest that the activation of *RFT1* expression in *Hd3a* RNAi plants is not induced by some unknown factor(s) associated with the RNAi method used in our study.

### A model for the regulation of photoperiodic flowering in rice

*Hd3a* is the main promoter of floral transition and flowering at about 60 DAS under SD conditions in wild-type plants. *RFT1*, like *Hd3a*, is regulated by *Hd1* under SD conditions, and its expression is diurnal with a peak at dawn (Fig. 1). Expression of *RFT1*, which is similar in time and space to *Hd3a* expression, is much lower than that of *Hd3a* (Figs 1, 2). However, when *Hd3a* expression is suppressed, as in *Hd3a* RNAi plants, *RFT1* expression increases at a later stage, and *RFT1* appears to complement or replace *Hd3a* as a floral activator (Figs 3, 4). The increase of *RFT1* expression induces the expression of two rice *API* orthologues, *OsMADS14* and *OsMADS15*, and also promotes flowering 30 days later than in wild-type plants (Fig. 5). Furthermore, when *RFT1* expression is activated in *Hd3a* RNAi plants, the level of H3K9 acetylation was higher than in wild-type plants, but not when *RFT1* expression is low (Fig. 6). This chromatin modification at the *RFT1* locus may lead to increased *RFT1* expression in *Hd3a* RNAi plants (Fig. 6). Suppression of both *Hd3a* and *RFT1* resulted in no flowering even 300 DAS (Fig. 3). These results indicate that *Hd3a* and *RFT1* are the major floral activators, and one or the other is essential for photoperiodic flowering in rice under SD conditions (Fig. 7). The molecular mechanism for *RFT1* expression in *Hd3a* RNAi plants and the function of *RFT1* under other environmental conditions remain to be studied. There may be an adaptive mechanism of plants to adjust to changes in the gene expression of a major regulator of flowering to secure flowering for the production of offspring.

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## Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/1135/4/767/DC1>

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