A gene network for long-day flowering activates \textit{RFT1} encoding a mobile flowering signal in rice

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Although some genes that encode sensory or regulatory elements for photoperiodic flowering are conserved between the long-day (LD) plant \textit{Arabidopsis thaliana} and the short-day (SD) plant rice, the gene networks that control rice flowering, and particularly flowering under LD conditions, are not well understood. We show here that \textit{RICE FLOWERING LOCUS T 1 (RFT1)}, the closest homolog to \textit{Heading date 3a (Hd3a)}, is a major floral activator under LD conditions. An RFT1:GFP fusion protein localized in the shoot apical meristem (SAM) under LD conditions, suggesting that \textit{RFT1} is a florigen gene in rice. Furthermore, mutants in OsMADS50, a rice ortholog of \textit{Arabidopsis SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)} did not flower up to 300 days after sowing under LD conditions, indicating that OsMADS50, which acts upstream of \textit{RFT1}, promotes flowering under LD conditions. We propose that both positive (OsMADS50 and Ehd1) and negative (Hd1, phyB and Ghd7) regulators of \textit{RFT1} form a gene network that regulates LD flowering in rice. Among these regulators, Ehd1, a rice-specific floral inducer, integrates multiple pathways to regulate \textit{RFT1}, leading to flowering under appropriate photoperiod conditions. A rice ortholog of \textit{Arabidopsis APETALA1} (\textit{OsAPETALA1}), \textit{OsMADS14}, was expressed in the floral meristem in wild-type but not in \textit{RFT1} RNAi plants, suggesting that \textit{OsMADS14} is activated by \textit{RFT1} protein in the SAM after the transition to flowering. We have thus exposed a network of genes that regulate LD flowering in rice.

KEY WORDS: Florigen, Photoperiodic flowering, Long-day conditions, Gene network, Rice

INTRODUCTION

Successful sexual reproduction in flowering plants depends on the accurate timing of flowering, which transits from vegetative stages to reproductive stages. Floral transition is regulated by both endogenous and environmental signals. Photoperiodic flowering is one of the most important factors in controlling floral transition among these various signals and is regulated both by day length and by the endogenous circadian rhythm (Thomas and Vince, 1977). Plants 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-day (SD) photoperiods, and day-neutral plants, which are not regulated by photoperiod. The signaling cascades of photoperiodic flowering have been extensively studied in \textit{Arabidopsis thaliana} (LDP) (Baurle and Dean, 2006; Imaizumi and Kay, 2006) and rice (SDP) (Izawa, 2007; Tuji et al., 2008). A number of signaling cascade genes have been identified and characterized. In \textit{Arabidopsis}, \textit{GIANTEA (GI)} integrates cellular signals from light sensory transduction and the circadian clock, and activates \textit{CONSTANS (CO)}, which encodes a zinc-finger transcriptional activator (Park et al., 1999; Samach et al., 2000). \textit{CO} induces \textit{FLOWERING LOCUS T (FT)}, which encodes a mobile flowering signal under LD conditions (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007; Mathieu et al., 2007). The \textit{GI–CO–FT} pathway is conserved in rice (OsGI-Hd1-Hd3a) (Yano et al., 2000; Kojima et al., 2002; Hayama et al., 2002). Expression of \textit{Hd3a}, the rice ortholog of \textit{FT}, is also induced by \textit{Ehd1}, a B-type response regulator that functions independently of \textit{Hd1} under SD conditions (Doi et al., 2004). \textit{OsMADS51}, which is regulated by OsGI, functions upstream of \textit{Ehd1} (Kim et al., 2007). It was recently reported that \textit{RID1/Ehd2/OsId1} is a positive regulator of both SD and LD flowering in rice (Wu et al., 2008; Matsubara et al., 2008; Park et al., 2008). By contrast, under LD conditions, \textit{Hd1} suppresses the expression of \textit{Hd3a} and causes delayed flowering (Hayama et al., 2003). \textit{Ghd7} encodes a transcription factor with a CCT motif, which acts as an LD-specific repressor of flowering (Xue et al., 2008). Thus, these studies revealed that rice flowering is regulated both by a ‘SD activation pathway’ and a ‘LD suppression pathway’ as an SDP. However, cultivated rice is grown extensively throughout Asia, and at the northern extremes of rice cultivation, including Japan and northern provinces of China and Korea, natural day length during rice cultivation is nearly LD (13-14.5 hours light) (Izawa, 2007), making LD flowering agronomically important in these regions. However, the genetic pathways governing LD flowering in rice are not well understood.

\textit{FT/Hd3a}, which is a common floral inducer in \textit{Arabidopsis thaliana} (LDP) and rice (SDP), encodes florigen, the mobile flowering signal (Tamaki et al., 2007; Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007; Mathieu et al., 2007), although the regulation of \textit{FT/Hd3a} expression differs with respect to day length to respond to the appropriate light conditions. In \textit{Arabidopsis}, \textit{TWIN SISTER OF FT (TSF)}, an FT homolog, promotes flowering redundantly with \textit{FT}, because \textit{ft–1; tsf–1} double mutants flower later than \textit{ft–1} single mutants (Michaels et al., 2004; Yamaguchi et al., 2005). \textit{RFT1} is the closest homolog of 13 \textit{FT-like} genes in rice to \textit{Hd3a}, with 91% identity in their deduced amino acid sequences and is located only 11.5 kb from \textit{Hd3a} on chromosome 6 (Kojima et al., 2002; Chardon and Damerval, 2005; Komiya et al., 2008). We previously reported that \textit{RFT1} promotes flowering in the absence of \textit{Hd3a}, and that both \textit{Hd3a} and \textit{RFT1} are essential for flowering.
under SD conditions, because double *RFT1-Hd3a* RNAi plants did not flower even at 300 days after sowing (Komiyama et al., 2008). In this study we analyzed the role of *RFT1* in LD flowering, and examined the possibility that a gene network is involved in rice LD flowering. We demonstrated that *RFT1* is essential for LD flowering and the *RFT1* protein is localized in the shoot apical meristem (SAM). Moreover, we showed that *Ehd1* acts as an integrator of multiple signals under LD conditions. Based on these results, we propose a model for the photoperiodic regulation of rice flowering consisting of both suppression and activation pathways.

**MATERIALS AND METHODS**

**Plant materials and growth conditions** *Japonica* rice cultivar (cv.) Norin 8 (N8) was used as wild type in RNAi analysis, and cv. Dongjin (DJ) was used as wild type for expression analyses of the OsMADS50 mutant. The T-DNA mutant of OsMADS50 was described previously (Lee et al., 2004). Plants were grown in climate chambers at 70% humidity under SD conditions with daily cycles of either 10 hours of light at 30°C and 14 hours of dark at 25°C, or under LD conditions with 14.5 hours of light and 9.5 hours of dark. Light was provided by fluorescent white light tubes (400 to 700 nm, 100 μmol m⁻² s⁻¹).

**RFT1 RNAi, Hd3a RNAi and pRFT1::RFT1:GFP constructs**

*RFT1* RNAi and *Hd3a* RNAi plants were described previously (Komiyama et al., 2008). *RFT1* cDNA (including 5'UTR) was amplified from Norin 8 rice cDNA with the primers *RFT1* 5'-F and *RFT1* 3'-R containing SpeI and XbaI sites and subcloned into Zero Blunt TOPO vector (Invitrogen) to construct pRFT1::RFT1:GFP (Table 1). A fragment of *RFT1* fused to the 3'UTR of *RFT1* by amplifying with GFP-F primer and *RFT1* 3'-R containing XbaI and SpeI sites was also subcloned into Zero Blunt TOPO vector (Invitrogen) (Table 1). The *RFT1* promoter region was amplified with *RFT1* pro-F and *RFT1* pro-R primer containing HindIII and Smal sites. To make the RFT1::GFP fusion constructs, fragments of NotI-SpeI-RFT1 cDNA-XbaI were inserted into the NotI-XbaI-GFP:3'UTR-RFT1-SpeI Blunt-TOPO vector (Table 1). There is a NotI restriction site inside Blunt-TOPO. SpeI fragments of RFT1:GFP:3'UTR RFT1 were inserted into the p2k+1 binary vector (Moriyama et al., 2005). Furthermore, a fragment of HindIII-RFT1 promoter-Smal was inserted into RFT1:GFP:3'UTR RFT1 p2k+1 binary vector. 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).

**RNA extraction and real-time PCR** Leaves were harvested at various times of the day, and total RNA was extracted using an RNAeasy plant mini kit (Qiagen), and treated with DNaseI (Invitrogen). cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen). One microliter of cDNA was used for the quantitative analysis of gene expression performed with SYBR Green PCR Master Mix (Applied Biosystems) with gene-specific primers (Table 1). Data were collected using the ABI PRISM 7000 sequence detection system in accordance with the instruction manual.

**GFP fluorescence** Transgenic rice plant tissues were visualized using a Zeiss LSM 510 Meta confocal laser scanning microscope. Rice plants were fixed in 6% agaroose (Nacalai Tesque). Transverse and longitudinal sections (50 μm) were then made using a vibrating-blade microtome (DOSAKA) and suspended in a drop of water on a covered glass slide. Fluorescence was excited with a 488 nm argon laser and emission images were collected in the 500-560 nm range. GFP signals were separated from background noise using an emission fingerprinting linear unmixing function.

**In situ hybridization** In situ hybridization was performed using the methods previously described (Kyozuka et al., 1998). The plasmids carrying full-length cDNAs were linearized and used as templates for making digoxigenin-labeled antisense probes.

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**Table 1. Primer sequences**

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**RESULTS**

*RFT1* and *Hd3a* regulate rice flowering under LD and SD conditions, respectively

To study the role of *RFT1* in LD flowering, we compared the flowering times of *Hd3a* RNAi plants and *RFT1* RNAi plants under LD conditions (14.5 hours light/9.5 hours dark). Although *Hd3a* RNAi plants flowered ~30 days later than wild-type plants under SD conditions (Fig. 1A), under LD conditions they flowered essentially at the same time as wild-type plants [116±3.6 days after sowing (DAS), n=4 for wild type versus 123±3.3 DAS, n=4 for *Hd3a* RNAi plants; Fig. 1B]. By contrast, *RFT1* RNAi plants flowered 213±38.8 DAS (n=12) under LD conditions, ~100 days later than wild type (Fig. 1B), whereas *RFT1* RNAi plants flowered slightly later than wild type under SD conditions (63±2.2 DAS, n=7 for wild type, 71±3.8 DAS, n=7 for *RFT1* RNAi plants; Fig. 1A). These results suggest that *RFT1* is the major floral activator under LD conditions and that *Hd3a* is the major floral activator under SD conditions in rice.
OsMADS50 has 50.6% amino acid sequence identity with SOC1, a flowering activator in Arabidopsis (Lee et al., 2000; Onouchi et al., 2000). A T-DNA insertion mutant of OsMADS50 flowers later than wild type under the natural light conditions of northeast Asia, which are similar to LD conditions (14 hours light/10 hours dark) (Lee et al., 2004). We investigated the flowering phenotype of OsMADS50 mutants under controlled day-length conditions and found that under LD conditions, wild-type plants (cv. Dongjin) flowered at 108±2.7 DAS, whereas the mutants failed to flower by 300 DAS (Fig. 1B). Under SD conditions, however, wild-type plants and the OsMADS50 mutant flowered within a week of each other (63±1.7 DAS, n=4 for wild type versus 69±3.5 DAS, n=4 for OsMADS50 mutants; Fig. 1A). The delay or inability of OsMADS50 mutants to flower only under LD conditions, much like that seen in RFT1 RNAi plants, indicates that OsMADS50 is also a positive regulator of flowering in response to LD.

The OsMADS50-Ehd1-RFT1 pathway is involved in floral activation under LD conditions

We next measured expression of OsMADS50 in leaves of RFT1 RNAi plants at 70 DAS under LD conditions, because SOC1 is positively regulated by FT in Arabidopsis (Yoo et al., 2005; Searle et al., 2006). OsMADS50 expression was not altered in RFT1 RNAi plants under LD conditions (Fig. 2A,G; see Fig. S1 in the supplementary material). By contrast, RFT1 expression was suppressed in leaves of the OsMADS50 mutant under LD conditions, suggesting that RFT1 acts downstream of OsMADS50 in leaves (Fig. 2B,H; see Fig. S1 in the supplementary material). We also examined the expression of other flowering-time genes in leaves of the OsMADS50 mutant. Ehd1, which encodes a rice-specific B-type response regulator, acts upstream of Hd3a family genes. Rice plants carrying a non-functional Ehd1 flower late under LD conditions, indicating that Ehd1 is an important factor for LD flowering (Doi et al., 2004). Ehd1 mRNA level was not altered in leaves in RFT1 RNAi plants, similarly to

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**Fig. 1.** RFT1 is a major LD activator and Hd3a is a major SD activator for rice flowering. (A,B) Days to flowering of Hd3a RNAi, RFT1 RNAi plants and OsMADS50 mutants under SD and LD conditions. Hd3a RNAi plants delay flowering under SD conditions (A), but not under LD conditions (B). Wild-type (cv. N8 and DJ) and Hd3a RNAi plants had already flowered at 120 DAS under LD conditions; RFT1 RNAi plants delayed flowering and flowered at about 210 DAS (B), but did not delay flowering under SD conditions (A). OsMADS50 mutants failed to flower through to 300 DAS under LD conditions (B), but the flowering time was the same as that of wild type under SD conditions (A). (C) Wild-type plants (cv. N8 and DJ), Hd3a RNAi plants, RFT1 RNAi plants and OsMADS50 mutants growing at 140 DAS under LD conditions. Wild-type plants and Hd3a RNAi plants flowered, but RFT1 RNAi plants and OsMADS50 mutants did not flower under LD conditions. Arrowheads show the panicle. DJ, Dongjin; Hi, Hd3a RNAi plants; M50, OsMADS50 mutants; N8, Norin 8; Ri, RFT1 RNAi plants.

**Fig. 2.** OsMADS50, an LD floral activator, acts upstream of Ehd1 and RFT1. (A-I) Diurnal expression of floral regulator genes in leaves of RFT1 RNAi (A,D,G) or OsMADS50 mutant (B,C,E,F,H,I) plants at 70 DAS (ZT 0: zeitgeber time) under LD conditions. In comparison to the expression of RFT1 (G), the expression of OsMADS50 (A) and Ehd1 (D) was not altered in RFT1 RNAi plants. OsMADS50 (B, Ehd1 (E), RFT1 (H) and Hd3a (I) were suppressed in OsMADS50 mutants, but Rid1/Ehd2/OslId1 (C) and Hd1 (F) expression levels were normal at 70 DAS under LD conditions. DJ, Dongjin; M50, OsMADS50 mutants; N8, Norin 8; Ri, RFT1 RNAi plants.
OsMADS50 mRNA (Fig. 2D). However, Ehd1 expression was lower in the OsMADS50 mutant, although expression of Hd1 and RID1/Ehd2/OsId1, which is an SD and LD floral activator (Wu et al., 2008; Matsubara et al., 2008; Park et al., 2008), was not altered (Fig. 2C,E,F; see Fig. S1 in the supplementary material). These results suggest that Ehd1 acts downstream of OsMADS50 and regulates RFT1 expression in leaves under LD conditions. As the expression of RID1/Ehd2/OsId1, which is an important activator of Ehd1 under LD conditions, was unchanged in OsMADS50 mutants (Fig. 2C) and OsMADS50 expression was not altered in OsId1 mutants (Park et al., 2008), it may regulate flowering independently of OsMADS50. Similarly, expression of Ghd7, an LD-specific suppressor of flowering (Xue et al., 2008), was not affected in the OsMADS50 mutant (data not shown), suggesting that it acts upstream of OsMADS50 or works in parallel to OsMADS50. Interestingly, Hd3a was also suppressed in leaves of the OsMADS50 mutant, suggesting that Hd3a is similarly regulated by OsMADS50 through Ehd1 under LD conditions (Fig. 2I; see Fig. S1 in the supplementary material). Therefore, Ehd1 regulates expression of both Hd3a and RFT1 in leaves, and promotes flowering under both SD and LD conditions (Doi et al., 2004).

Hd3a and RFT1 are both diurnally expressed with a peak at dawn, with a gradual decrease in expression during the day under SD conditions (Izawa et al., 2002; Hayama et al., 2003; Komiya et al., 2008). Under LD conditions, RFT1 expression was also diurnal, with a peak at dawn (Fig. 2G,H). Moreover, expression patterns of OsMADS50 and Ehd1 were similar to that of RFT1 under LD conditions (Fig. 2A,B,D,E), suggesting that OsMADS50, Ehd1 and RFT1 are regulated by the circadian clock.

The expression analysis in leaves indicates that OsMADS50 is not regulated by RFT1 in leaves and that the OsMADS50-Ehd1-RFT1 pathway is involved in floral activation under LD conditions. To study the regulation of LD floral genes in the SAM, we analyzed the expression of OsMADS50 and RFT1 in the SAM at various developmental stages, from vegetative stage to floral organ initiation. The expression level of OsMADS50 was very low in the SAM through all stages analyzed (Fig. 3A). Furthermore, in situ hybridization analysis showed that OsMADS50 mRNA was not detected in the SAM at around 70 DAS in either wild-type or RFT1 RNAi plants under LD conditions, although it was detected in the stem in wild-type and RNAi plants (Fig. 3D,E). OsMADS50 expression was not detected in the floral meristem around the secondary panicle branch initiation (Fig. 3F). In rice the level of OsMADS50 mRNA is high in leaves; however, it is very low in the SAM at any developmental stage. These results suggest that OsMADS50 regulates expression of Ehd1 and RFT1 in rice leaves and that it is not significantly expressed in the SAM. RFT1 expression was suppressed in leaves of the OsMADS50 mutants under LD conditions, suggesting that RFT1 acts downstream of OsMADS50 in leaves of rice.

**RFT1 encodes a major LD florigen**

Hd3a encodes a mobile flowering signal, or florigen, under SD conditions in rice (Tamaki et al., 2007). To examine whether RFT1 protein is a florigen, the 1.8 kb RFT1 promoter was fused to a RFT1:GFP construct to form pRFT1::RFT1:GFP, and introduced into rice plants by Agrobacterium-mediated transformation (Fig. 4A). The RFT1 promoter, like Hd3a, is active in leaf blades under SD conditions (Tamaki et al., 2007; Komiya et al., 2008). In pRFT1::RFT1:GFP transgenic rice plants, expression of RFT1:GFP is much higher than endogenous RFT1 in wild-type plants, and those transgenic rice plants flowered earlier than wild-type plants under LD conditions, suggesting that expression of RFT1:GFP induces early flowering (see Fig. S2 in the supplementary material). To determine the localization of RFT1 protein in pRFT1::RFT1:GFP plants under LD conditions, we measured GFP fluorescence around the SAM with confocal laser scanning microscopy. RFT1:GFP was detected in the SAM and vascular tissue (Fig. 4B,C). The RFT1:GFP signal was also detected in the meristem after floral transition (Fig. 4D,E).

The presence of RFT1 mRNA in the shoot apex during the transition from vegetative to floral organ initiation stage was examined under LD conditions. RFT1 mRNA was virtually absent from the SAM, suggesting that there is little or no RFT1 promoter activity in the SAM (Fig. 3B), as had been shown with Hd3a (Tamaki et al., 2007). These results indicate that RFT1 is transcribed in leaf blades under LD conditions and that RFT1 moves from leaves to the SAM through phloem and promotes LD floral transition, as Hd3a does under SD conditions.
OsMADS14 and OsMADS15 act downstream of RFT1 in the SAM under LD conditions

In Arabidopsis, expression of APETALA1 (API) is induced by FT in the SAM (Abe et al., 2005; Wigge et al., 2005). In rice, OsMADS14 and OsMADS15, which are rice orthologs of API, are suppressed in leaves of Hd3a RNAi plants under SD conditions (Komiya et al., 2008). OsMADS14 and OsMADS15 are upregulated after the transition to the reproductive phase in the floral meristem, when it begins to differentiate into primary panicle branch primordia (Furutani et al., 2006) (Fig. 3C; see Fig. S3 in the supplementary material). To identify genes acting downstream of RFT1 under LD conditions, we examined expression of OsMADS14 and OsMADS15 in leaves of RFT1 RNAi, Hd3a RNAi plants and OsMADS50 mutants. Expression of OsMADS14 and OsMADS15 was suppressed in RFT1 RNAi plants and OsMADS50 mutants, but not in Hd3a RNAi plants, which did not alter the flowering date under LD conditions (Fig. 5A-C; see Fig. S3 in the supplementary material). These results indicate that OsMADS14 and OsMADS15 act downstream of RFT1 in leaves under LD conditions.

To study the effect of RFT1 protein on OsMADS14 expression in the SAM, in situ hybridization was performed. OsMADS14 RNA was not detected in the SAM at the vegetative stage in wild-type plants (Fig. 5D). However, OsMADS14 mRNA was observed in the floral meristem at the stages of primary panicle branch initiation and secondary panicle branch initiation after floral transition in wild type under LD conditions (Fig. 5F,H). By contrast, in RFT1 RNAi plants, OsMADS14 mRNA was not detected in the SAM at three developmental stages examined: 70, 90 and 200 DAS (Fig. 5E,G,J). These results suggest that RFT1 protein promotes floral transition in the SAM and induces activation of OsMADS14 and OsMADS15 in the floral meristem directly or indirectly. Although OsMADS15 expression in the SAM was not analyzed by in situ hybridization because its expression in the SAM was much lower than OsMADS14 (Fig. 3C; see Fig. S3 in the supplementary material), it is likely to be regulated similarly by RFT1 in the SAM.

Ehd1 integrates multiple signals for LD flowering in rice

Long-day suppression of rice flowering becomes apparent when the photoperiod is longer than 13 hours (Nishide et al., 2004). To analyze effects of light duration on flowering time and on the expression of flowering-related genes, plants were grown under a light regimen of either LD14/10 (14 hours light/10 hours dark) or LD14.5/9.5 (14.5 hours light/9.5 hours dark). Japonica rice cultivar Norin 8 grown under LD14.5/9.5 conditions flowered 20 days later than those grown under LD14/10 (Fig. 6A), and cv. Dongjin grown under LD14.5/9.5 conditions flowered 13 days later than LD14/10 conditions (see Fig. S4 in the supplementary material). These results indicate that the 30 minute difference under LD conditions was sufficient to substantially delay flowering. Expression of Ehd1, RFT1 and Hd3a in leaves was also decreased under the longer light conditions (Fig. 6B-D), suggesting that Ehd1 expression is suppressed by light under LD conditions. By contrast, expression of OsMADS50 and Hd1 in both cv. Norin 8 and Dongjin was not affected by the 30 minute extension of light (see Fig. S4 in the supplementary material).
Phytochrome B (phyB), which codes for a plant photoreceptor, negatively regulates rice flowering under natural light conditions, which are similar to LD (Takano et al., 2005), and Hd3a mRNA expression is suppressed by light via the phyB receptor pathway (Ishikawa et al., 2005). Therefore, we examined the effects of phyB mutants on the expression of genes involved in LD flowering in leaves. phyB-3 mutants flowered ~20 days earlier than wild type under LD14.5/9.5 conditions (14.5 hours light/9.5 hours dark) conditions. Expression of Ehd1, RFT1 and Hd3a in the phyB-3 mutants was strongly increased relative to that of the wild type (Fig. 6F-H). However, the expression of OsMADS50, Hd1, Ghd7 and RID1/OsId1/Ehd2 was not affected by the phyB mutation (Fig. 6I-L). Although Ghd7 suppresses Ehd1 and acts as a floral suppressor specifically under LD conditions (Xue et al., 2008), its suppression of Ehd1 seems to be independent of phyB-mediated inhibition. Therefore, under LD conditions, Ehd1 is downregulated by phyB and Ghd7, and upregulated by OsMADS50 and RID1/Ehd2/OsId1. As Ehd1 receives multiple signals from light and from other genes, and transmits them to RFT1 for flowering, Ehd1 may be considered as an integrator for LD floral initiation signals.

**DISCUSSION**

Based on the current study and previous rice floral transition studies, we would like to propose a model to explain the network of positive and negative regulators of the photoperiodic flowering in rice (Fig. 7). Only one SD activation pathway is known. Hd3a, which is activated by Hd1 and Ehd1, promotes flowering and induces OsMADS14 and OsMADS15 expression. When Hd3a expression is knocked down by RNAi (marked by X in Fig. 7), RFT1 becomes activated later during development for SD flowering (Komiya et al., 2008). No suppression pathway for SD flowering is known. Under LD conditions, both activation and suppression pathways are involved in regulation of flowering. Hid1 suppresses Hd3a expression under LD conditions (Hayama et al., 2003). Thus, OsGI-Hd1-Hd3a is an LD suppression pathway. We showed that Ehd1 and RFT1 are also suppressed by phyB in this study.

Our results indicate that OsMADS50 and RFT1 are key positive regulators of flowering under LD conditions, because RFT1 RNAi plants and OsMADS50 mutants mainly delay LD flowering but not SD flowering. Furthermore, we showed that OsMADS50 acts upstream of RFT1 in leaves under LD conditions. RNA analysis of overexpressing and loss-of-function mutants of OsMADS50 in leaves showed that OsMADS50 acts upstream of Hd3a, OsMADS14 and OsMADS15 (Lee et al., 2004). In situ hybridization and developmental analysis of OsMADS50 expression in the SAM showed that OsMADS50 mRNA was not detected in the SAM or the floral meristem of wild type or RFT1 RNAi plants (Fig. 3). These results suggest that in rice OsMADS50, which acts upstream of RFT1, regulates LD-flowering in leaves, but this regulation is different from the analogous one in Arabidopsis (Yoo et al., 2005; Searle et al., 2006). RFT1 expression is positively regulated in leaves by OsMADS50 and Ehd1 under LD conditions. RFT1 protein produced in leaves moves from the leaves to the SAM and promotes floral transition. Expression of OsMADS14 and OsMADS15 increased from the time of panicle branch initiation in the floral meristem (Figs 3 and 5). These results suggest that floral transition is induced by the RFT1 protein in the SAM, and that after the transition OsMADS14 and OsMADS15 initiate formation of the floral organs.

Under LD conditions, levels of RFT1 and Hd3a mRNAs gradually increased from 50 DAS to 100 DAS and the level of RFT1 mRNA was only slightly higher than that of Hd3a at 75 DAS, concurrent with floral transition (see Fig. S5 in the supplementary material). Thus, the difference in the expression level could not explain the difference in the function of these two genes. One possible explanation for the difference in the function of these two genes is that the functions of Hd3a and RFT1 in different photoperiodic conditions may be regulated at the protein level. For instance, interactors required for the floral induction with RFT1 in LD might be different from those with Hd3a in SD, but both proteins activate the same downstream regulators, OsMADS14 and OsMADS15. A more detailed study of gene expression and protein localization before and after floral transition in the SAM will be required to fully understand the molecular mechanisms of RFT1 function in the SAM.

RID1/Ehd2/OsId1 is a positive regulator of Ehd1, but as expression of OsMADS50 in OsId1 mutants and RID1/Ehd2/OsId1 in OsMADS50 mutants is not altered, RID1/Ehd2/OsId1 appears to regulate Ehd1 independently of OsMADS50. Ehd1 expression is both positively and negatively regulated in leaves by multiple factors. OsMADS50 mutants did not flower at 300 days under LD 14.5/9.5 (14.5 hours light/9.5 hours dark) conditions, though these mutants flowered at 166±17.7 days under LD 14/10 conditions (data not shown). The extension of light for 30 minutes under LD conditions delayed flowering of OsMADS50 mutants. Together, these results suggest that LD flowering is regulated by two opposing functions: suppression by light and activation by the OsMADS50 pathway. Furthermore, PhyB acts as a negative regulator of LD-flowering...
through suppression of *Ehd1* expression. **OsMADS50**, *Ghd7*, *phyB* and *RID1/Ehd2/OsId1* are all involved in the regulation of *Ehd1*, indicating that it integrates multiple signals in LD flowering.

The northern limit for the growth of wild species of rice is around 28°N, but cultivated rice is grown even at 45°N in northern Asia (Izawa et al., 2007). Rice cultivation in northeastern Asia thus requires earlier flowering so that grain is set before the cold season arrives. Rice cultivars were developed with a weak SD response and the ability to flower under LD conditions (Izawa et al., 2007). **RFT1** may have arisen by tandem duplication of *Hd3a* after the divergence of rice from a progenitor cereal because **RFT1** is unique to rice among cereals, and the physical distance between **RFT1** and *Hd3a* is present in the *Oryza rufipogon* genome. The nucleotide sequence analysis of **RFT1** and *Hd3a* in *O. rufipogon* and *O. sativa* indicate that **RFT1** has diverged more rapidly than *Hd3a* during rice evolution (Hagiwara et al., 2009), that **RFT1** might have acquired function for LD flowering during the process of rice breeding and that **RFT1** gained an LD florigen function to adapt to environments in northern Asia. It was shown that non-functional or weaker *Ghd7* alleles are grown around 35-45°N in northern Asia, whereas functional *Ghd7* alleles are found in tropical rice (<32°N) (Xue et al., 2008). These results suggest that the loss or decreased function of *Ghd7* floral suppressor may be another factor acquired for the spread of rice cultivation in northern Asia. It was recently demonstrated that *Ehd1* expression levels contribute to diversity of flowering time in cultivated rice under SD conditions, although *Ehd1* protein function is highly conserved (Takahashi et al., 2009). Thus, the diversity of **OsMADS50** could possibly influence the diversity of *Ehd1* expression levels under LD conditions. This remains to be studied in the future.

Although **RFT1** RNAi plants did not flower even in November under natural conditions in Nara, Japan (34°N), wild-type and *Hd3a* RNAi plants flowered by this time (data not shown), indicating that **RFT1** is a key gene for the promotion of flowering under natural conditions in northern Asia. Rice plants deficient in both **RFT1** and *Hd3a* did not flower even at 300 DAS under either SD or LD conditions (Komiyama et al., 2008) (and data not shown), strongly suggesting that **RFT1** and *Hd3a* are the only genes for rice florigen. Understanding of the molecular mechanisms involved in the activation of **RFT1** and *Hd3a* and the function of *Hd3a* as an SD florigen and **RFT1** as an LD florigen will be an important subject of future studies.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/20/3443/DC1

**References**


