

# A gene network for long-day flowering activates *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 *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 *RICE FLOWERING LOCUS T 1* (*RFT1*), the closest homolog to *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 *RFT1* is a florigen gene in rice. Furthermore, mutants in *OsMADS50*, a rice ortholog of *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 *RFT1*, promotes flowering under LD conditions. We propose that both positive (*OsMADS50* and *Ehd1*) and negative (*Hd1*, *phyB* and *Ghd7*) regulators of *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 *RFT1*, leading to flowering under appropriate photoperiod conditions. A rice ortholog of *Arabidopsis APETALA1*, *OsMADS14*, was expressed in the floral meristem in wild-type but not in *RFT1* RNAi plants, suggesting that *OsMADS14* is activated by *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 *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 *Arabidopsis*, *GIGANTEA* (*GI*) integrates cellular signals from light sensory transduction and the circadian clock, and activates *CONSTANS* (*CO*), which encodes a zinc-finger transcriptional activator (Park et al., 1999; Samach et al., 2000). *CO* induces *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 *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 *Hd3a*, the rice ortholog of *FT*, is also induced by *Ehd1*, a B-type response regulator that functions independently of *Hd1* under SD conditions (Doi et al., 2004). *OsMADS51*, which is regulated by *OsGI*, functions upstream of *Ehd1* (Kim et al., 2007). It was recently reported that *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, *Hd1* suppresses the expression of *Hd3a* and causes delayed flowering (Hayama et al., 2003). *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.

*FT/Hd3a*, which is a common floral inducer in *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 *FT/Hd3a* expression differs with respect to day length to respond to the appropriate light conditions. In *Arabidopsis*, *TWIN SISTER OF FT* (*TSF*), an *FT* homolog, promotes flowering redundantly with *FT*, because *ft-1*; *tsf-1* double mutants flower later than *ft-1* single mutants (Michaels et al., 2004; Yamaguchi et al., 2005). *RFT1* is the closest homolog of 13 *FT-like* genes in rice to *Hd3a*, with 91% identity in their deduced amino acid sequences and is located only 11.5 kb from *Hd3a* on chromosome 6 (Kojima et al., 2002; Chardon and Damerval, 2005; Komiya et al., 2008). We previously reported that *RFT1* promotes flowering in the absence of *Hd3a*, and that both *Hd3a* and *RFT1* are essential for flowering

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under SD conditions, because double *RFT1-Hd3a* RNAi plants did not flower even at 300 days after sowing (Komiya 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

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

*RFT1* RNAi and *Hd3a* RNAi plants were described previously (Komiya et al., 2008). *RFT1* cDNA (including 5'UTR) was amplified from Norin 8 rice cDNA with the primers *RFT1* 5'-F and *RFT1* 5'-R containing *SpeI* and *XbaI* sites and subcloned into Zero Blunt TOPO vector (Invitrogen) to construct *pRFT1::RFT1:GFP* (Table 1). A fragment of *GFP* 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 *SmaI* 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 (Moritoh et al., 2005). Furthermore, a fragment of *HindIII-RFT1* promoter-*SmaI* 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 RNeasy plant mini kit (Qiagen), and treated with DNaseI (Invitrogen). cDNA was synthesized from 1  $\mu\text{g}$  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% agarose (Nacalai Tesque). Transverse and longitudinal sections (50  $\mu\text{m}$ ) 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 (Koyuzuka et al., 1998). The plasmids carrying full-length cDNAs were linearized and used as templates for making digoxigenin-labeled antisense probes.

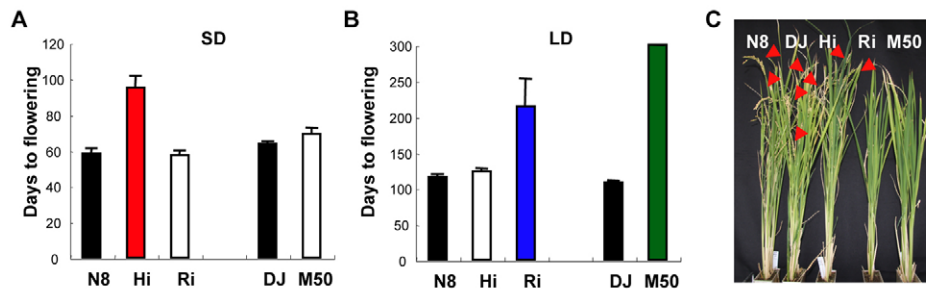
**Table 1. Primer sequences**

Name	Sequence (59 to 39)
<b>Expression analysis</b>	
<i>Ubq</i> -F	AACCAGCTGAGGCCCAAGA
<i>Ubq</i> -R	ACGATTGATTAACCAGTCCATGA
<i>OsMADS50</i> -F	CAGGCCAGGAATAAGCTGGAT
<i>OsMADS50</i> -R	TTAGGATGGTTGGTGCATTGC
<i>Ehd1</i> -F	TGCAAATGGCGCTTTTGAT
<i>Ehd1</i> -R	ATATGTGCTGCCAAATGTTGCT
<i>RFT1</i> -F	TGACCTAGATTCAAAGTCTAATCCTT
<i>RFT1</i> -R	TGCCGGCCATGTCAAATTAATAAC
<i>Hd3a</i> -F	GCTCACTATCATCATCCAGCATG
<i>Hd3a</i> -R	CCTTGCTCAGCTATTTAATTGCATAA
<i>OsMADS14</i> -F	CGGTGCGAGACGAGGAA
<i>OsMADS14</i> -R	GAAAGACGGTGCTGGACGAA
<i>OsMADS15</i> -F	CGTCGTCGGCCAAACAG
<i>OsMADS15</i> -R	TGACTTCAATTCATTCAAGGTTGCT
<i>Hd1</i> -F	TCAGCAACAGCATATCTTCTCATCA
<i>Hd1</i> -R	TCTGGAATTTGGCATATCTATCACC
<i>Ghd7</i> -F	ATGGGGATGGCCAATGAGGAGTC
<i>Ghd7</i> -R	GAGGAATCCGGCCGCTTTTTTC
<i>RID1/Ehd2/OsID1</i> -F	CGACGACAATAGCTCGATCGC
<i>RID1/Ehd2/OsID1</i> -R	GTGCATGGTCACGGAGCCTT
<b>RNAi constructs</b>	
<i>Hd3a</i> -F	TACTTCAACTGCCAGCGGAGGCAG
<i>Hd3a</i> -R	TGCTGGATGATGATAGTGAGCATGC
<i>RFT1</i> -F	TACTTCAACTGCCAGCGGAGG
<i>RFT1</i> -R	AGCTATAGCTGCTGCATGCATGGA
<b>pRFT1::RFT1:GFP</b>	
<i>RFT1</i> promoter-F	AAGCTTTGATATTCTCGACCCAGTCTTGCT
<i>RFT1</i> promoter-R	CCCGGGGATGCACTAGTTGTGCAAGCTTCTC
<i>RFT1</i> 5'-F	ACTAGTCTGTCACTGTTGGCTAGCTTA
<i>RFT1</i> 5'-R	GCGGCAGGAGGGTCTACCCCTCTAGA
<i>GFP</i> -F	TCTAGATCTAGAGTGAGCAAGGGCGA
<i>RFT1</i> 3'-R	ACTAGTTATACAGCTAGGCAGGTCTCAG
<b>Screening pRFT1::RFT1:GFP transgenic plants</b>	
<i>GFP2</i> -F	TACGGCGTGCACTGCTTC
<i>GFP2</i> -R	CGGGCATGGCGGACT
<b>Screening RNAi plants</b>	
<i>Hd3a</i> -F	GTCTACTTCAACTGCCAGCGGAG
<i>Hd3a</i> -R	GAACCTGCAATGTATAGCATGCTGG
<i>RFT1</i> -F	GTCTACTTCAACTGCCAGCGGAG
<i>RFT1</i> -R	CTTAGCTATAGCTGCTGCATGCATG

## 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 [ $116 \pm 3.6$  days after sowing (DAS),  $n=4$  for wild type versus  $123 \pm 3.3$  DAS,  $n=4$  for *Hd3a* RNAi plants; Fig. 1B]. By contrast, *RFT1* RNAi plants flowered  $213 \pm 38.8$  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 ( $63 \pm 2.2$  DAS,  $n=7$  for wild type,  $71 \pm 3.8$  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.

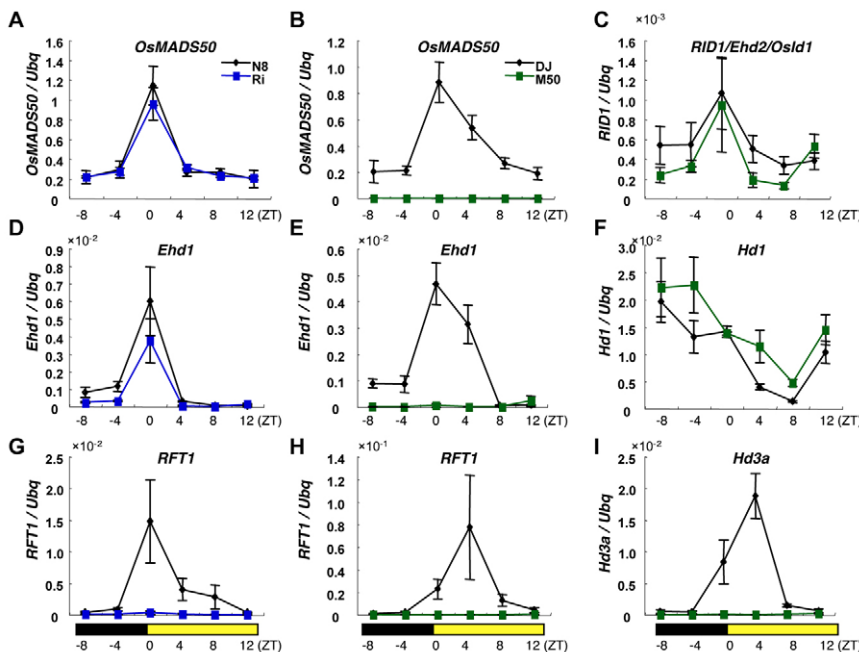


**Fig. 1. *RFT1* is a major LD activator and *Hd3a* is a major SD activator for rice flowering.** (A,B) Days to flowering of *Hd3a* RNAi, *RFT1* RNAi plants and *OsMADS50* mutants under SD and LD conditions. *Hd3a* RNAi plants delay flowering under SD conditions (A), but not under LD conditions (B). 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.

*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  $108 \pm 2.7$  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 ( $63 \pm 1.7$  DAS,  $n=4$  for wild type versus  $69 \pm 3.5$  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



**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/Osld1* (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.



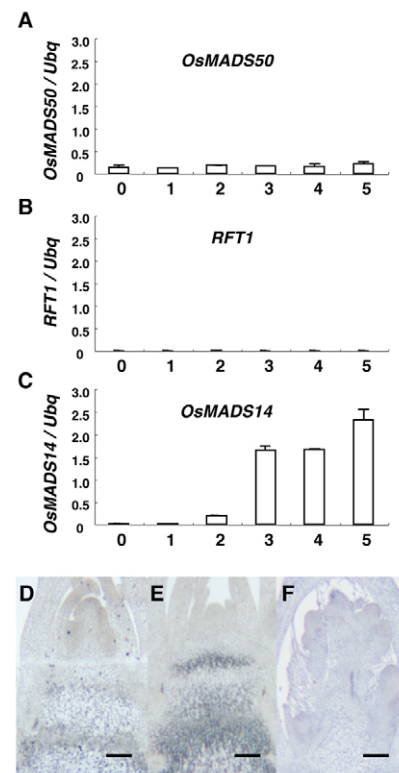
*OsMADS50* mRNA (Fig. 2D). However, *Ehd1* expression was lower in the *OsMADS50* mutant, although expression of *Hd1* and *RID1/Ehd2/OsId1*, which is an SD and LD floral activator (Wu et al., 2008; Matsubara et al., 2008; Park et al., 2008), was not altered (Fig. 2C,E,F; see Fig. S1 in the supplementary 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

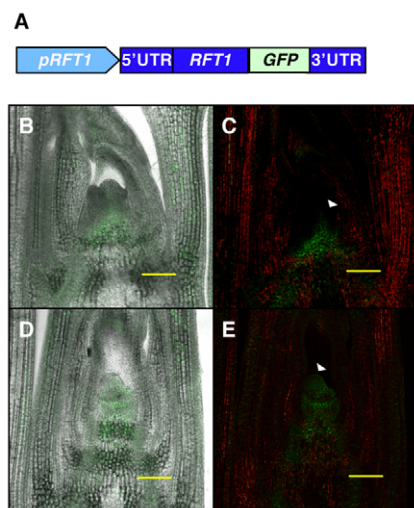


**Fig. 3. Expression of *OsMADS50*, *RFT1* and *OsMADS14* in the SAM.**

(A-C) Developmental expression of *OsMADS50*, *RFT1* and *OsMADS14* in the SAM under LD conditions through stages 1-5. Expression of *OsMADS50* and *RFT1* was very low in the SAM throughout the plant's life cycle (A,B). Expression of *OsMADS14* increased in the inflorescence meristem starting from the primary panicle branch-generating stage (stage 2, C). Japonica rice cv. Shiokari was used for these analyses. cDNA samples from SAM mRNA in wild-type plants were generated as previously described (Furutani et al., 2006). (D-F) In situ hybridization patterns of *OsMADS50* in the SAM and floral meristem. *OsMADS50* expression at 70 DAS in wild-type (D) and *RFT1* RNAi plants (E) under LD conditions. *OsMADS50* expression was not detected at the SAM in wild-type plants (D) or *RFT1* RNAi plants (E). *OsMADS50* expression was not detected in floral meristem of wild-type plants (F). Scale bars: 100  $\mu$ m. Stages: 0, SAM shortly before transition to reproductive development; 1, early stage of primary panicle branch initiation; 2, late stage of primary panicle branch initiation; 3, secondary panicle branch initiation; 4, spikelet meristem initiation; 5, floral organ initiation.

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.

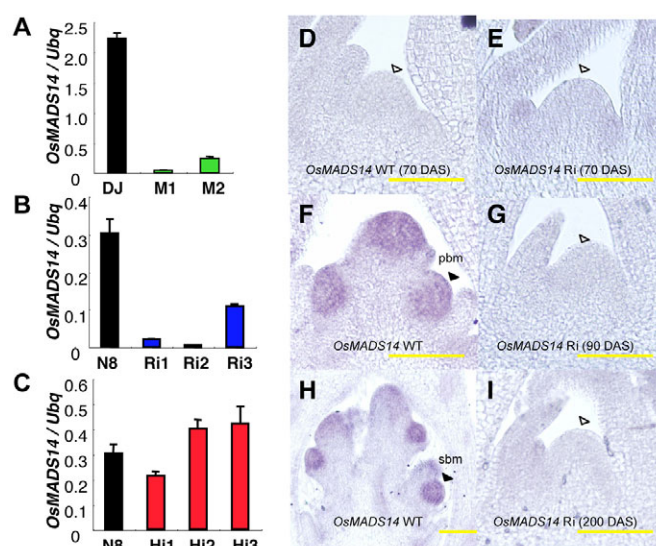


**Fig. 4. *RFT1* encodes LD florigen.** (A) *pRFT1::RFT1::GFP* constructs. *RFT1* promoter, 1.8 kb, 5'UTR of *RFT1*+*RFT1* and *GFP* fused 3'UTR of *RFT1* were used for the *pRFT1::RFT1::GFP* constructs. (B-E) Confocal images of *pRFT1::RFT1::GFP* transgenic plants. Longitudinal sections through the SAM of vegetative stages (B,C) and floral meristem after floral transition (D,E). B,D are composite images of the fluorescein isothiocyanate (FITC) and transmission channels. (C,E) Spectrally unmixed images. *RFT1::GFP* fluorescence is shown in green, and plant autofluorescence is in red. Arrowheads indicate the SAM. Scale bars: 100 μm.

### ***OsMADS14* and *OsMADS15* act downstream of *RFT1* in the SAM under LD conditions**

In *Arabidopsis*, expression of *APETALA1* (*AP1*) 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 *AP1*, 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,I). 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



**Fig. 5. In situ hybridization patterns of *OsMADS14* in the SAM.**

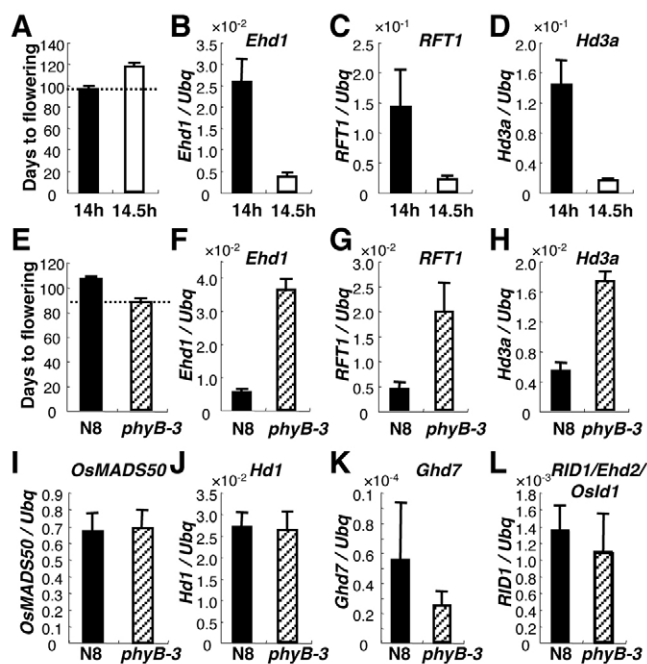
(A-C) Expression of *OsMADS14* in leaves of *OsMADS50* mutants, *RFT1* RNAi and *Hd3a* RNAi plants at 70 DAS (ZT 0: zeitgeber time) under LD conditions. Expression of *OsMADS14* was suppressed in the leaves of *OsMADS50* mutants (M1, M2; A) and *RFT1* RNAi plants (Ri1, Ri2, Ri3; B). However, expression of *OsMADS14* was not altered in *Hd3a* RNAi plants (Hi1, Hi2, Hi3; C), which flowered at the same time as wild-type plants (cv. DJ and N8). (D-I) *OsMADS14* expression in wild-type plants and *RFT1* RNAi plants under LD conditions. *OsMADS14* was not detected in the SAM at 70 DAS, shortly before transition to reproductive development in wild-type plants under LD conditions (D). *OsMADS14* mRNA was observed in the floral meristem at the stages of primary panicle branch initiation (F) and secondary panicle branch initiation (H) in wild-type plants. *OsMADS14* mRNA was not detected in the SAM at 70 (E), 90 (G) or 200 DAS (I) in *RFT1* RNAi plants. White arrows show the SAM; black arrowheads indicate the pbm and the sbm. Scale bars: 100 μm. pbm, primary panicle branch meristem; sbm, secondary panicle branch meristem. DJ, Dongjin; N8, Norin 8.

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).





**Fig. 6. *Ehd1* expression is suppressed by light through *phyB*.**

(A) Days to flowering under LD14/10 (14 hours light/10 hours dark) and LD14.5/9.5 (14.5 hours light/9.5 hours dark) conditions. (B–D) Expression of *Ehd1* (B), *RFT1* (C) and *Hd3a* (D) in leaves at 70 DAS (ZT 0: zeitgeber time) under LD14/10 (14 hours light/10 hours dark) and LD14.5/9.5 (14.5 hours light/9.5 hours dark) conditions. (E) Days to flowering in *phyB-3* mutants under LD14.5/9.5 conditions. (F–H) Expression of *Ehd1* (F), *RFT1* (G) and *Hd3a* (H) was higher in *phyB-3* mutants than in wild type at 50 DAS under LD conditions. Expression of *OsMADS50* (I), *Hd1* (J), *Ghd7* (K) and *RID1/Ehd2/OsId1* (L) was not altered.

*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* 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 (Fig. 6E), suggesting that *phyB* acts as a negative regulator of LD flowering. 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

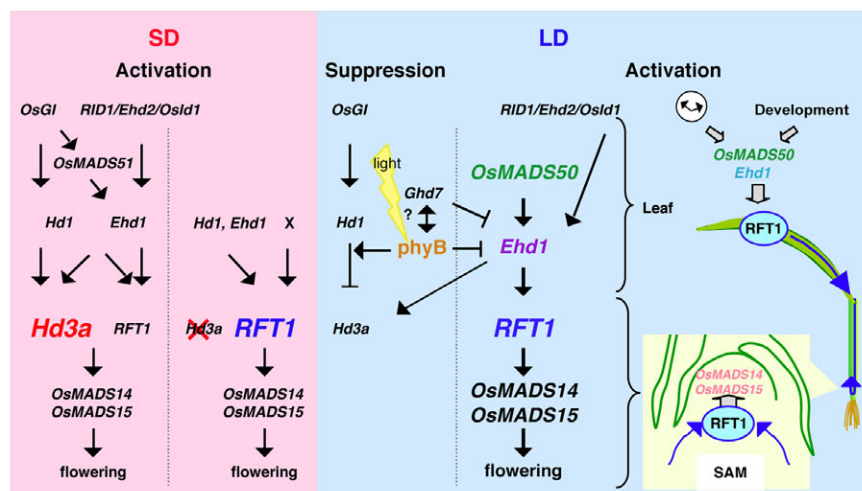
Based on the current study and previous rice floral transition studies, we would like to propose a model to explain the network of positive and negative regulators of the photoperiodic flowering in rice (Fig.

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. *Hd1* 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



**Fig. 7. A model for the photoperiodic control of flowering in rice.** Under SD conditions, *Hd1* and *Ehd1* positively regulate *Hd3a* and promote flowering in the activation pathway. When *Hd3a* expression is knocked down by RNAi (marked by X), *RFT1* becomes activated later during development for SD flowering. No suppression pathway for SD flowering is known. Under LD conditions, both activation and suppression pathways are involved in regulation of flowering. *Hd1* suppresses *Hd3a* expression under LD conditions. Thus, *OsGI-Hd1-Hd3a* is an LD suppression pathway. *RFT1*, which encodes a floral mobile signal, promotes LD flowering. *RFT1* expression is positively regulated in leaves by *OsMADS50* and *Ehd1* and *phyB* suppresses *Ehd1* and *RFT1* expression. *Ghd7* suppresses *Ehd1* expression under LD conditions. As *Ehd1* is positively and negatively regulated by multiple factors, it can be considered to be an integrator of multiple flowering signals in leaves under LD conditions. *RFT1* protein produced in leaves moves from leaves to the SAM and promotes floral transition. Expression of *OsMADS14* and *OsMADS15* is activated by *RFT1* in the SAM.

through suppression of *Ehd1* expression. *OsMADS50*, *Ghd7*, *phyB* and *RID1/Ehd2/Osld1* 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 around 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 only 11.5 kb (Chardon and Damerval, 2005; Komiya et al., 2008). *RFT1* 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 (Komiya et al., 2008) (and data not shown), strongly suggesting that *RFT1* and *Hd3a* are the only genes for rice florigens. Understanding of the molecular mechanisms involved in the activation of *Hd3a* and *RFT1* 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>

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