OsMADS51 Is a Short-Day Flowering Promoter That Functions Upstream of *Ehd1*, *OsMADS14*, and *Hd3a*[1][W][OA]

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Although flowering regulatory mechanisms have been extensively studied in Arabidopsis (*Arabidopsis thaliana*), those in other species have not been well elucidated. Here, we investigated the role of *OsMADS51*, a type I MADS-box gene in the short-day (SD) promotion pathway in rice (*Oryza sativa*). In SDs *OsMADS51* null mutants flowered 2 weeks later than normal, whereas in long days loss of *OsMADS51* had little effect on flowering. Transcript levels of three flowering regulators—*Ehd1*, *OsMADS14*, and *Hd3a*—were decreased in these mutants, whereas those of *OsGI* and *Hd1* were unchanged. Ectopic expression of *OsMADS51* caused flowering to occur about 7 d earlier only in SDs. In ectopic expression lines, transcript levels of *Ehd1*, *OsMADS14*, and *Hd3a* were increased, but those of *OsGI* and *Hd1* remained the same. These results indicate that *OsMADS51* is a flowering promoter, particularly in SDs, and that this gene functions upstream of *Ehd1*, *OsMADS14*, and *Hd3a*. To further investigate the relationship with other flowering promoters, we generated transgenic plants in which expression of *Ehd1* or *OsGI* was suppressed. In *Ehd1* RNA interference plants, *OsMADS51* expression was not affected, supporting our conclusion that the MADS-box gene functions upstream of *Ehd1*. However, in *OsGI* antisense plants, the *OsMADS51* transcript level was reduced. In addition, the circadian expression pattern for this MADS-box gene was similar to that for *OsGI*. These results demonstrate that *OsMADS51* functions downstream of *OsGI*. In summary, *OsMADS51* is a novel flowering promoter that transmits a SD promotion signal from *OsGI* to *Ehd1*.

The ability to adjust the timing of flowering is an important factor when trying to adapt plants to regional environments and control their harvest time. Regulatory mechanisms that control flowering have been studied extensively in Arabidopsis (*Arabidopsis thaliana*). In this long-day (LD) species, flowering is promoted by several regulatory genes, including *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), and *APETALA1* (*AP1*).

GI encodes a nuclear protein that defines the proper amplitude and period length of circadian rhythms (Park et al., 1999; Mizoguchi et al., 2005). In Arabidopsis, mutations in *GI* delay flowering in LDs, but have little or no effect in short days (SDs). *GI* expression is regulated by the circadian clock, with a peak in transcript levels at 8 to 10 h after dawn. Timing, height, and duration of this peak are influenced by daylength (Fowler et al., 1999). The GI protein acts between the circadian oscillator and CO to promote flowering by increasing *CO* and *FT* mRNA abundance (Mizoguchi et al., 2005). CO is a putative transcription factor containing two B-box zinc fingers and a C-terminal CCT domain (Putterill et al., 1995). *CO* promotes flowering in response to LDs but not SDs (Putterill et al., 1995). CO protein activates the expression of several early target genes (e.g. *SOC1* and *FT* [Samach et al., 2000; Suarez-Lopez et al., 2001]). *FT*, which encodes a protein similar to phosphatidylethanolamine-binding proteins and Raf kinase inhibitor proteins, also promotes flowering in LDs (Kardailsky et al., 1999; Kobayashi et al., 1999). FT is primarily expressed in vascular tissues, whereas its interactor, FD, is predominantly expressed at the shoot apex (Abe et al., 2005; Wigge et al., 2005). *SOC1* integrates vernalization and GA signals for flowering (Moon et al., 2005). Its expression is affected by the loss of *FT*, whereas activation-tagged *FT* mutants have been shown to induce *SOC1* up-regulation (Schmid et al., 2003; Moon et al., 2005). In contrast, *FT* expression remains unaffected by either loss or gain of *SOC1* expression. In response to FT-mediated floral induction, AP1 contributes to the inflorescence meristem becoming committed to flowering (Huang et al., 2005).

In contrast to Arabidopsis, rice (*Oryza sativa*) is a SD plant in which flowering is inhibited during early

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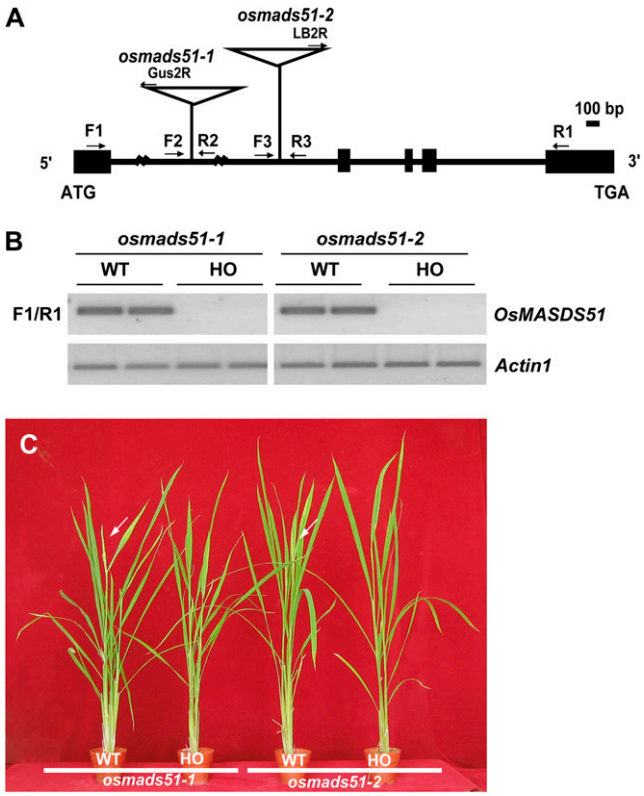

Figure 1. *OsMADS51* genomic structure, positions of T-DNA insertional mutant alleles, and *osmads51* mutant phenotypes. A, Schematic diagram of T-DNA insertional position on *OsMADS51*. This gene consists of five exons (black boxes) and four introns (lines between black boxes). T-DNA is located at 9,606 bp (*osmads51-1*) and 15,927 bp (*osmads51-2*) from translation start site. F1 and R1 are primers used for RT-PCR and real-time PCR analyses. F2, R2, and Gus2R are primers for genotyping *osmads51-1*. F3, R2, and LB2R are primers for genotyping *osmads51-2*. B, Semiquantitative RT-PCR of *OsMADS51* transcript in *osmads51* mutants (HO) and wild-type segregants (WT). *Actin1* was used for normalization of cDNA quantity. PCR cycles numbered 28 in *OsMADS51* and 25 in *Actin1*. C, Phenotype of *OsMADS51* mutants (HO) compared with wild-type segregants (WT). Plants were cultivated in a growth room under SD conditions (10 h light/14 h dark). Photograph was taken at 75 DAG. Arrows indicate panicles emerging at heading stage.

developmental stages, but induced when the day-length shortens in the fall. Although the roles of genes involved in the photoperiod pathway are generally conserved between rice and Arabidopsis, some differences exist. *OsGI*, a *GI* ortholog, regulates flowering time in response to photoperiodic conditions. Mutations in this gene delay flowering in inductive SDs, whereas flowering is marginally induced under LDs (Hayama et al., 2003). In comparison, overexpression of *OsGI* in transgenic rice causes late flowering in both SDs and LDs. Expression of *Hd1*, the ortholog of *CO*, is affected in these plants, which indicates that *OsGI* functions to regulate *Hd1*, a gene that also shows circadian rhythm (Hayama et al., 2003). However, its expression pattern differs from that of *OsGI*. *Hd1* transcript levels are lowest at around midday and become high during the night (Izawa et al., 2002; Kojima et al., 2002; Hayama et al., 2003). In contrast to *CO*, *Hd1* has a double role in regulating downstream flowering activators—repression in LDs and promotion in SDs (Yano et al., 2000). One of the downstream targets is *Hd3a*, a *FT* ortholog, which acts preferentially in inductive SDs (Kojima et al., 2002). In addition to this photoperiod-controlled pathway, rice contains an alternative inductive pathway that appears to function independently of *Hd1*. *Ehd1*, encoding a B-type response regulator, promotes flowering by inducing the expressions of *Hd3a*, *FTL1*, and *OsMADS14* (Doi et al., 2004). These results indicate that two different signaling pathways are intrinsically involved in the photoperiodic flowering pathway in rice.

The relationship among AP1 group MADS-box genes seems to differ between rice and Arabidopsis. For example, *AP1* and its related genes determine floral meristem identity downstream of *FT*. However, in rice, *OsMASD14*, a member of the AP1 group, apparently functions upstream of *Hd3a* as a flowering activator. Its ectopic expression causes significant early flowering in tissue culture (Jeon et al., 2000), and transcription of *Hd3a* is up-regulated in *OsMADS14*-overexpressing cultured cells (S. Lee and G. An, unpublished data).

Most plants eventually flower, even under nonpermissive conditions. This autonomous pathway is regulated mainly by *SOC1* in Arabidopsis. The gene acts as a flowering inducer, a result of converging photoperiod, autonomous, vernalization, and GA pathways (Araki, 2001; Mouradov et al., 2002; Simpson and Dean, 2002). *OsMASD50*, the Arabidopsis *SOC1* homolog, acts as a flowering activator similar to *SOC1*. Ectopic expression causes dramatic early flowering at the regenerating callus stage, and *OsMASD50* mutants flower about a month late (Lee et al., 2004; Lee and An, 2007).

Most MADS-box genes that regulate flowering time belong to type II, a group that contains the conserved MEF2-like MADS-box and K regions. In addition, intermediate (I) and C-terminal (C) regions are present. Therefore, they are termed MIKC-type genes (Munster et al., 1997). The possible roles of the other group, type I MADS-box genes in flowering time, are not well understood. Type I group proteins contain a conserved SRF-like MADS domain, but the C-terminal region is not clearly defined. Furthermore, type I proteins do not carry the conserved K-box region (Alvarez-Buylla

Table I. *Days to heading in osmads51*

Days to heading was scored when the first panicle appeared. WT, Wild type; HO, *osmads51*/*osmads51* homozygotes.

Alleles	Genotypes	Days to Heading under SD	Days to Heading under LD
osmads51-1	WT	75 ± 2.2	96 ± 3.8
	HO	89 ± 5.2	100 ± 3.2
osmads51-2	WT	75 ± 2.2	97 ± 2.5
	HO	90 ± 3.8	101 ± 4.0

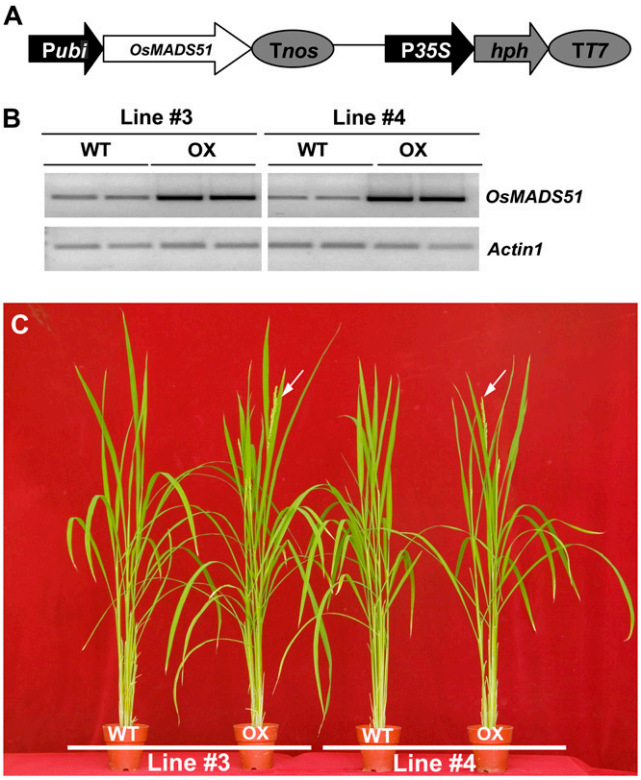

Figure 2. *OsMADS51* overexpression. A, Schematic diagram of *Ubi*::*OsMADS51* construct. *OsMADS51* full-length cDNA clone was placed between *Ubi* promoter (P*ubi*) and *nos* terminator (T*nos*). B, Semiquantitative RT-PCR analyses of *Ubi*::*OsMADS51* transgenic (OX) lines 3 and 4 and their segregating wild-type controls (WT). *Actin1* was used for normalization of cDNA quantity. RT-PCR was performed over 28 cycles for *OsMADS51* and 25 cycles for *Actin1*. C, Phenotype *Ubi*::*OsMADS51* transgenic plants (OX) and their segregating wild-type controls (WT) cultivated for 62 d under SD conditions. Arrows indicate heading panicles.

et al., 2000). Although overexpression of a type I MADS-box gene, *AGAMOUS-LIKE28* (*AGL28*), promotes flowering within the autonomous pathway in Arabidopsis (Yoo et al., 2006), the roles of most MADS-box genes of that type remain unknown.

Here, we have elucidated the functional role of a type I MADS-box gene, *OsMADS51*, in rice. This gene was originally identified through large-scale cDNA analysis within the rice genome research program (Shinozuka et al., 1999). *OsMADS51* is unique to rice; a homologous gene is lacking in Arabidopsis. However, homologous genes do occur, with 74% identity in wheat (*Triticum aestivum*; accession no. ABF57941) and maize (*Zea mays*; accession no. CAD23412). Nevertheless, their roles have not yet been elucidated. OsMADS51 is composed of 164 amino acids and contains an SRF-like MADS domain. However, like other type I MADS-box genes, the K-box region is not conserved and the C-terminal region is short. Expression is ubiquitous in all organs (Shinozuka et al., 1999). In this study, we have investigated the function of *OsMADS51* with regard to flowering time.

RESULTS

Isolation of T-DNA Insertional Mutants in *OsMADS51*

We have previously reported the generation of a T-DNA insertional mutant population in japonica rice (Jeon and An, 2001; Jeong et al., 2002, 2006; An et al., 2003, 2005b; Ryu et al., 2004). The sequence database is open to the public (www.postech.ac.kr/life/pfg/risd) and seeds are available to the scientific community. From that database, we have identified two independent T-DNA insertional mutant alleles, *osmads51-1* and *osmads51-2*, in which T-DNAs are inserted into the first intron in both alleles, at 9,606 and 15,927 bp from the start codon in *osmads51-1* and *osmads51-2*, respectively (Fig. 1A). Homozygous (*osmads51*/*osmads51*) plants did not accumulate *OsMADS51* transcript (Fig. 1B). Under SDs, flowering in the homozygous progeny of both alleles was about 2 weeks later than in wild-type segregants (Fig. 1C; Table I). In contrast, little change in flowering time was noted under LDs (Table I). These results indicate that *OsMADS51* is a flowering promoter that functions preferentially in SDs.

Ectopic Expression of *OsMADS51* Causes Early Flowering

To further elucidate the functional roles of *OsMADS51*, we constructed a chimeric binary vector (*Ubi*:*OsMADS51*) by placing a *OsMADS51* full-length cDNA under the maize *ubiquitin* (*Ubi*) promoter (Fig. 2A). This construct was introduced into *Agrobacterium* and transferred into plant cells via cocultivation (An et al., 1985; Hiei et al., 1997). Seven primary (T_1) transgenic plants were obtained and planted in a paddy field. All showed early flowering, albeit to varying degrees. For example, two lines flowered 2 weeks early, three lines about 10 d sooner, and two lines by just about 3 d compared with our control transgenic plants. The extent of early flowering was correlated with *OsMADS51* expression levels when confirmed by northern-blot analyses (data not shown).We then chose two lines (3 and 4) that manifested early flowering by 10 d and selected homozygous plants and segregating wild type using hygromycin selection. T_3 progeny were reared in a growth room in either SDs (10 h light) or LDs (14 h light) to elucidate the

Table II. *Days to heading in OsMADS51-overexpressing plants*

Days to heading was scored when the first panicle appeared. WT, Wild type; OX, homozygous *Ubi*::*OsMADS51*.

Transgenic Plants	Genotypes	Days to Heading under SD	Days to Heading under LD
Line 3	WT	72 ± 3.8	93 ± 3.1
	OX	63 ± 4.0	91 ± 2.4
Line 4	WT	73 ± 1.7	94 ± 2.4
	OX	66 ± 2.3	93 ± 3.3

daylength-dependent roles of *OsMADS51*. Homozygous plants exhibited early flowering by 9 d (line 3) and 7 d (line 4) in SDs. However, no significant phenotypic difference was observed between homozygous transgenic lines and wild-type plants grown in LDs (Table II). These results support our conclusion that *OsMADS51* is a SD flowering activator.

Relationship of *OsMADS51* with Various Flowering-Time Regulators

To evaluate the relationship between *OsMADS51* and previously identified flowering regulators, we investigated the expression of *OsGI*, *Hd1*, *Ehd1*, *OsMADS14*, and *Hd3a* in the *osmads51* mutants and their wild-type segregants grown in continuous SDs or LDs. At 55 d after germination (DAG), leaf blades were harvested and transcript levels were measured by reverse transcription (RT)-PCR and quantitative real-time PCR (Fig. 3; Supplemental Fig. S1). Expression of *OsGI* and *Hd1* was unchanged in the *OsMADS51* mutants under both lighting conditions (Fig. 3, A, B, D, and E). However, transcript levels of *Ehd1*, *OsMADS14*, and *Hd3a* were greatly reduced in the *osmads51* mutants in SDs and LDs (Fig. 3, A, B, F, G, and H). These results indicate that *Ehd1*, *Hd3a*, and *OsMADS14* probably

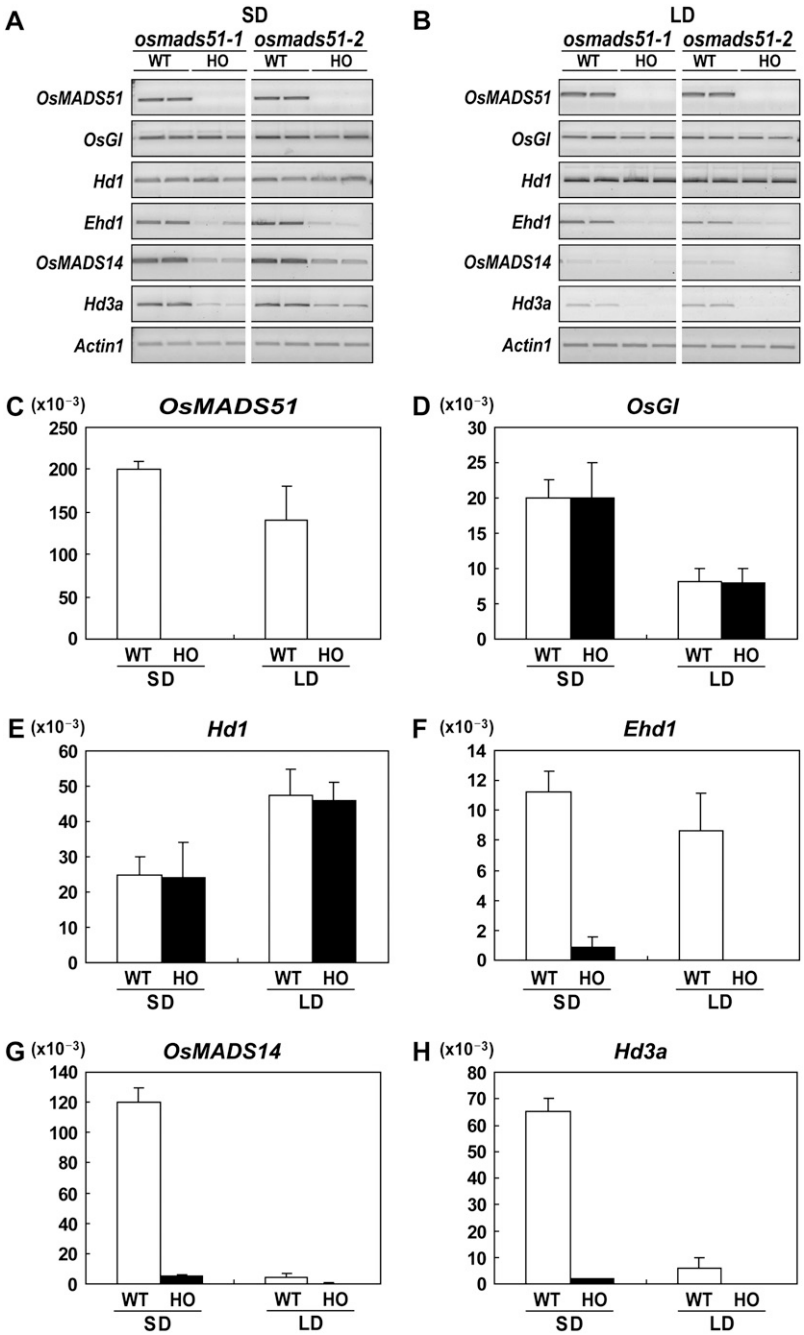

Figure 3. Expression profiles for various flowering regulators in *OsMADS51* mutants. Shown are semi-quantitative RT-PCR analyses of *OsMADS51*, *OsGI*, *Hd1*, *Ehd1*, *OsMADS14*, and *Hd3a* in wild-type (WT) and *OsMADS51* mutant plants (HO) grown for 55 d under SD (A) or LD (B) conditions. *Actin1* was used for normalization of cDNA quantity. RT-PCR cycles numbered 28 for all flowering regulators and 25 for *Actin1*. From at least three independent experiments, the most reproducible data are presented. Real-time PCR analyses of *OsMADS51* (C), *OsGI* (D), *Hd1* (E), *Ehd1* (F), *OsMADS14* (G), and *Hd3a* (H) in WT and HO plants grown for 55 d under SD or LD conditions. RNA was prepared from leaf blades harvested at 0 h after light was turned on. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

function downstream of *OsMADS51*. As expected, the transcript levels of *OsMADS51*, *OsGI*, *Ehd1*, *OsMADS14*, and *Hd3a* were higher in SD-grown plants. Interestingly, this expression difference between SDs and LDs was greatest for *OsMADS14* and *Hd3a*. Therefore, it appears that LD-specific inhibitors coordinately regulate these downstream genes. *Hd1* is one candidate because it has been proposed to be a negative regulator in LDs (Yano et al., 2000), and we find its transcript level is higher in LDs compared with SDs.

Transcript levels were also examined from these five genes in overexpressing *OsMADS51* transgenic plants (Fig. 4; Supplemental Fig. S2). We used 35-DAG plants because they had flowered early and, therefore, their regulatory genes were expressed earlier. In wild-type control plants, transcript levels of *Ehd1*, *OsMADS14*, and *Hd3a* were very low at 35 DAG in LDs (Fig. 4), whereas transcripts were detected at 55 DAG (Fig. 3), showing age-dependent up-regulation patterns. As expected from the knockout experiment, *Ehd1*, *Hd3a*, and *OsMADS14* transcript levels increased (Fig. 4, A, B, F, G, and H) under SD conditions, whereas those of *OsGI* and *Hd1* were not significantly changed in the *Ubi*::*OsMADS51* transgenic plants in either daylength

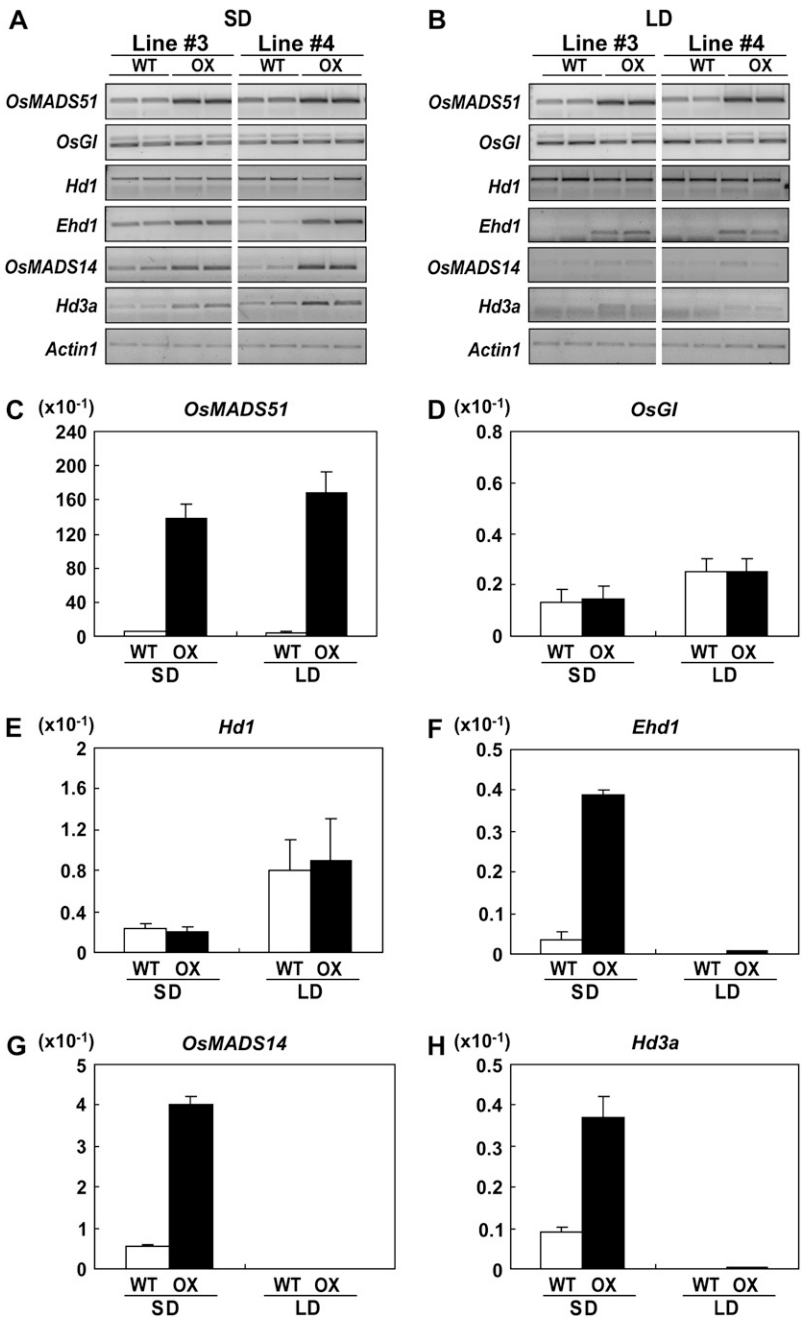

Figure 4. Expression profiles for various flowering regulators in *OsMADS51*-overexpressing lines. Shown are semiquantitative RT-PCR analyses of *OsMADS51*, *OsGI*, *Hd1*, *Ehd1*, *OsMADS14*, and *Hd3a* in WT and *Ubi*::*OsMADS51* plants (OX) grown for 35 d under SD (A) or LD (B) conditions. *Actin1* was used for normalization of cDNA quantity. RT-PCR cycles numbered 28 for all flowering regulators under SD; 32 for *Ehd1*, *OsMADS14*, and *Hd3a*; and 28 for *OsMADS51*, *OsGI*, and *Hd1* under LD. For *Actin1*, 25 cycles were performed. From at least three independent experiments, the most reproducible data are presented. Real-time PCR analyses of *OsMADS51* (C), *OsGI* (D), *Hd1* (E), *Ehd1* (F), *OsMADS14* (G), and *Hd3a* (H) in WT and *OsMADS51* mutant plants (HO) grown for 35 d under SD or LD conditions. RNA was prepared from leaf blades harvested at 0 h after light was turned on. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

(Fig. 4, A, B, D, and E). These results support our conclusion that *OsMADS51* functions upstream of *Ehd1*, *Hd3a*, and *OsMADS14*.

Expression Analyses of *OsMADS51* in *OsGI* Antisense Plants

In *osmads51* mutants and *Ubi*::*OsMADS51* transgenic plants, transcript levels of *OsGI* were unchanged (Figs. 3 and 4). Therefore, we believe that *OsGI* either functions upstream of *OsMADS51* or is part of an independent pathway. Hayama et al. (2003) have previously reported that *OsGI* RNAi plants show delayed flowering in SDs, and we observed the same late-flowering phenotype in our *OsGI* antisense plants. Therefore, this SD-specific effect suggests that *OsGI* and *OsMADS51* may function within the same pathway. To investigate the relationship between these two genes, we generated *OsGI* antisense plants and found that the *OsMADS51* transcript level was reduced in plants grown under either SDs or LDs (Fig. 5A). As controls, we also examined expression levels from other flowering regulators—*Ehd1*, *OsMADS14*, and *Hd3a*—that had been demonstrated to function downstream of *OsGI* (Hayama et al., 2003; Doi et al., 2004). As expected, their transcript levels were reduced in our *OsGI* antisense plants (Fig. 5, E–G). These results indicate that *OsGI* does function in the same pathway as *OsMADS51*, with *OsGI* occurring upstream.

Expression Analyses of *OsMADS51* in *Ehd1*-RNAi Plants

In *osmads51* plants, the *Ehd1* transcript level was significantly reduced (Fig. 3, A and F), whereas it was notably increased in the *Ubi*::*OsMADS51* plants (Fig. 4, A and F). Therefore, it is likely that *Ehd1* is located downstream of *OsMADS51*. To confirm this hypothesis, we generated transgenic plants carrying the *Ehd1*-RNA interference (RNAi) construct (Fig. 6B). In all, 30 independently transformed plants were obtained, among which 20 flowered later than the control. We observed that *Ehd1* transcript levels were reduced in those transgenics that were delayed. Two RNAi plants were selected that showed the most significant change in their flowering time. Under our SD conditions, they flowered about 7 d later than controls. *Ehd1* transcript

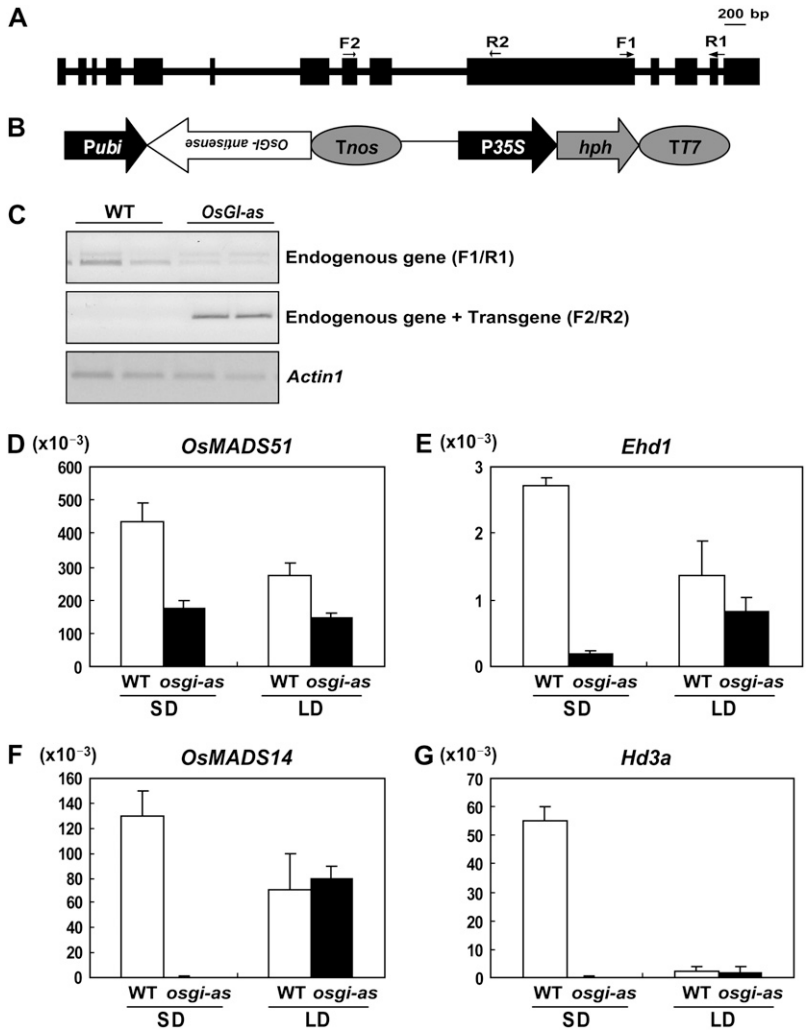

Figure 5. Expression level analyses of flowering regulators in *OsGi* antisense plants. A, Schematic diagram of *OsGi*. F1, F2, R1, and R2 are primers used for RT-PCR and real-time PCR analyses. B, Schematic diagram of *OsGi* antisense construct. C, RT-PCR analysis of *OsGi* antisense (*OsGi-as*) plants and their wild-type segregants (WT). *OsGi-as* plants were grown for 55 d under SDs or LDs. RNA was prepared from leaf blades harvested at 0 h after light was turned on. Endogenous *OsGI* transcript was amplified with F1 and R1 primer sets, and both *OsGI-as* and endogenous transcripts were amplified with F2 and R2 primers. PCR cycles numbered 28 for *OsGI* and 25 for *Actin1*. *Actin1* was used for normalization of cDNA quantity. Transcript amounts for *OsMADS51* (D), *Ehd1* (E), *OsMADS14* (F), and *Hd3a* (G) were analyzed by quantitative real-time PCR. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

levels were reduced in those late-flowering transgenic plants in which a significant amount of RNAi transcript accumulated (Fig. 6C). Because the primers used for detecting the RNAi transcript amplified the endogenous *Ehd1* transcript as well, we also found a PCR band in the wild type.

In the *Ehd1*-RNAi plants, *OsGI* and *OsMADS51* transcript levels were not changed (Fig. 6, D and E), confirming that they function upstream of *Ehd1*. As previously reported (Doi et al., 2004), expression of *Hd3a* was significantly reduced in our *Ehd1*-RNAi plants (Fig. 6G). The *OsMADS14* transcript level was also reduced in the RNAi plants, confirming the possibility that these flowering regulators are downstream of *OsGI* (Fig. 6F).

Diurnal Rhythms of *OsMADS51* and *OsGI*

Because *OsMAD51* functions between *OsGI* and *Ehd1*, the transcript level of a MADS-box gene may oscillate as do *OsGI* and *Ehd1*. Therefore, we measured *OsMADS51* transcription in leaf blades from 55-DAG plants grown under SDs or LDs (Fig. 7). As reported previously (Hayama et al., 2003), the *OsGI* transcript level was low at the beginning of the day (Fig. 7A), but increased under light and reached a maximum at the end of the day. In darkness, that level was continuously reduced to a minimal amount. A similar rhythm was observed in LDs (data not shown). Under SDs, a similar induction curve was observed for *OsMADS51* (Fig. 7B), increasing under light and decreasing in the dark. However, the change in transcript amounts was not as great as that for *OsGI*. Under LD, no such rhythm was found (data not shown).

Relationship between *OsMADS51* and *OsMADS50*

The relationship between *OsMADS51* and *OsMADS50* was also investigated. We have previously reported that *osmads50* plants show a late-flowering phenotype (delay of about 1 month), whereas overexpressors of *OsMADS50* display an extreme early-flowering phenotype (Lee et al., 2004). Here, our mutations downregulated the expression of *OsMADS14* and *Hd3a*, suggesting that *OsMADS50* functions upstream of *OsMADS14*. To evaluate whether *OsMADS50* is another flowering activator that may influence *OsMADS51*, we examined whether *OsMADS51* expression was altered

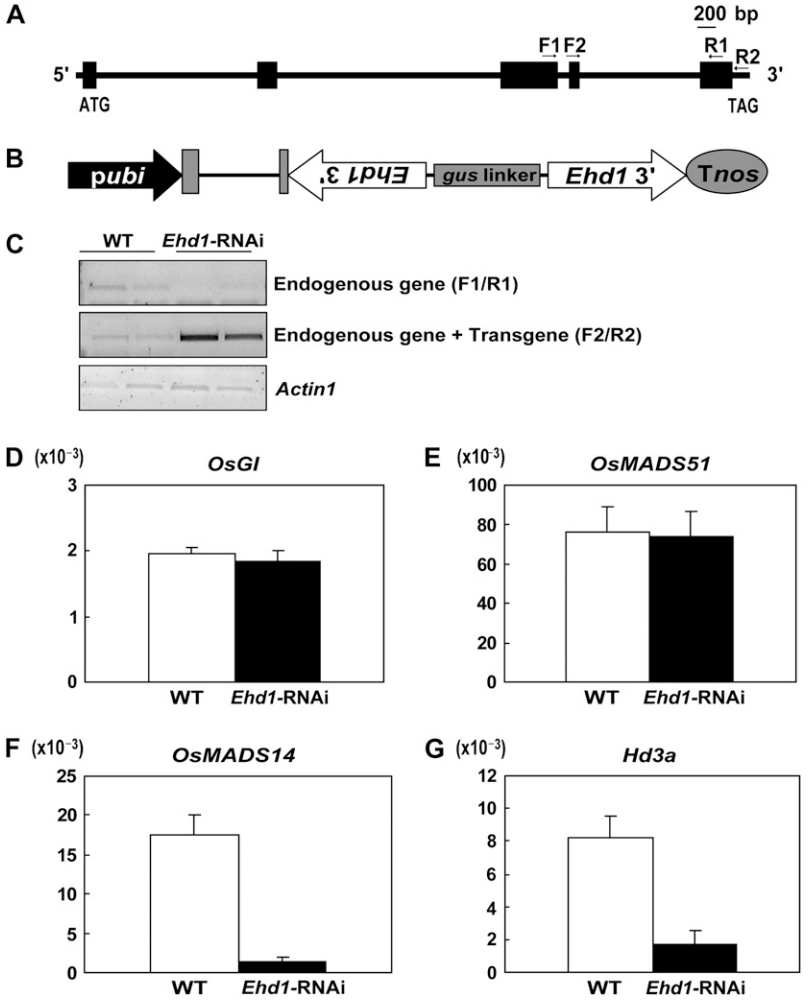

Figure 6. Expression level analysis of flowering regulators in *Ehd1*-RNAi plants. A, Schematic diagram of *Ehd1*. F1, F2, R1, and R2 are primers used for RT-PCR and real-time PCR analyses. B, Schematic diagram of *Ehd1*-RNAi construct. C, RT-PCR analysis of *Ehd1*-RNAi plants and their wild-type segregants (WT). Endogenous *Ehd1* transcript was amplified with F1 and R1 primer sets and both *Ehd1*-RNAi and endogenous transcripts were amplified with F2 and R2 primers. PCR cycles numbered 28 for *Ehd1* and 25 for *Actin1*. *Actin1* was used for normalization of cDNA quantity. D to G, Real-time PCR analysis of *OsMADS51* (D), *OsGI* (E), *OsMADS14* (F), and *Hd3a* (G) in *Ehd1*-RNAi plants. Samples for RT-PCR and real-time PCR were harvested at 0 h after light was turned on. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

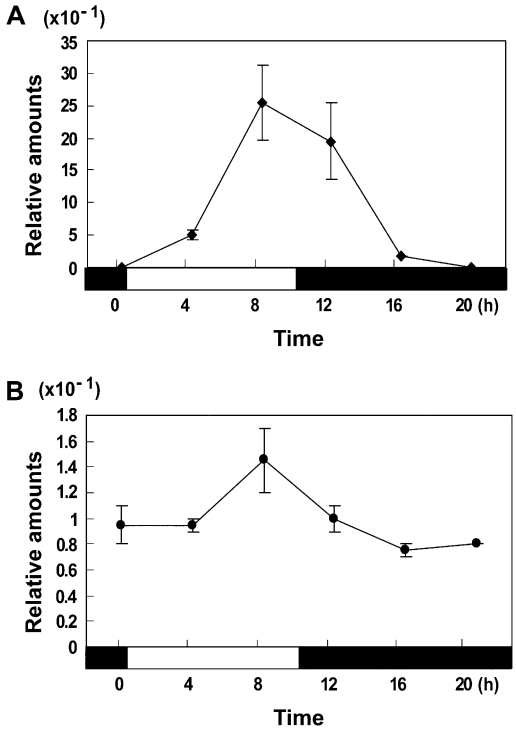

Figure 7. Diurnal rhythms of *OsMADS51* (A) and *OsGI* (B) under SD conditions. Plants were grown under SDs of 10 h light (0–10 h, white box) and 14 h dark (10–24 h, black box). RNA was prepared from leaf blades of 50-DAG plants at 0, 4, 8, 12, 16, and 20 h after light was turned on. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

in the *osmads50* plants. However, that proved not to be the case (Supplemental Fig. S3). We also found that levels of *OsMADS50* were unchanged in the *osmads51* plants (Supplemental Fig. S3). Our results indicate that these two MADS-box genes function independently to control flowering time.

Analysis of Transcript Levels of *OsMADS51* According to Developmental Stage

Because *OsMADS51* is an early-acting activator, we measured its transcript levels at various developmental stages to see whether they would remain constant or change as plants matured. At 0 h (immediately after the light was turned on), we sampled leaf blades from plants grown in SDs. Real-time PCR analyses showed that transcription was high during the early developmental stages, up to day 43, but then declined and remained low over the remaining period (Fig. 8). This indicates that *OsMADS51* functions early in the first 2 months of growth in rice.

DISCUSSION

Intensive molecular genetics studies on the regulatory mechanisms that control flowering time have revealed that some major players are well conserved between rice and Arabidopsis (Izawa et al., 2003). However, some regulatory factors are found in just one of these model systems. For example, *FLC* is present only in Arabidopsis, whereas *Ehd1* is exclusive to rice (Michaels and Amasino, 1999; Doi et al., 2004). Therefore, understanding the mechanisms that regulate flowering involves focusing on those species-specific genes to gain essential information that will elucidate the details of this diversification between species. In fact, the existence of the *FLC* family in Arabidopsis makes it possible to explain the vernalization pathway in crucifers, which does not exist in rice (Michaels and Amasino, 1999).

We, therefore, speculated that some MADS-box genes have been diversified to control gene-specific flowering mechanisms. *OsMADS51* was selected for investigation in this regard because MADS-box genes of this group are found only in grass species. OsMADS51 was first identified via differential analysis of transcripts from mature spikelets (Ciaffi et al., 2005). Although the expression pattern for *OsMADS51* has already been studied (Shinozuka et al., 1999), our report concerns the functional roles for this gene.

Our transgenic analyses showed that *OsMADS51* is an SD-dependent flowering activator. Under SDs, *OsMADS51* mutants flower about 2 weeks later compared with the control, whereas transformants that overexpress *OsMADS51* flower 1 to 2 weeks early. Under LDs, these effects are milder, with *OsMADS51* mutants flowering 3 to 4 d later and the overexpressors flowering no more than 1 to 2 d early. In rice, two SD promotion pathways have been proposed—one mediated by *Hd1* and the other by *Ehd1* (Yano et al., 2000; Doi et al., 2004). These pathways converge to induce *Hd3a* and the resultant protein product is moved to the shoot apical meristem to induce flowering (Tamaki

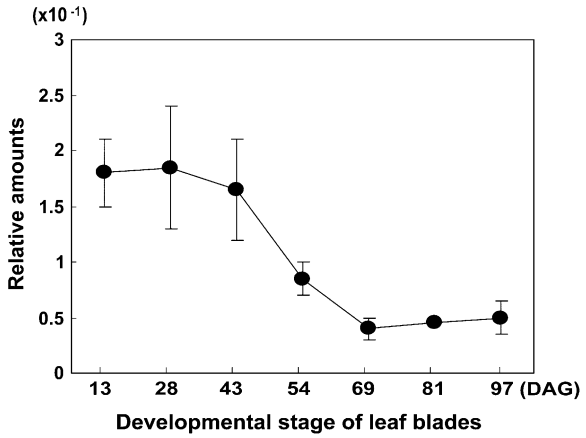

Figure 8. Changes in *OsMADS51* transcript levels during growth phase. Plants were reared under SD conditions (10 h light/14 h dark). RNA was prepared from leaf blades of 13-, 28-, 43-, 54-, 69-, 81-, and 97-DAG plants at 0 h after light was turned on. Each data point is the average of two or more independent experiments. *y* axis, Relative values between transcript levels of regulatory genes and *Ubi*; vertical bars, standard deviation.

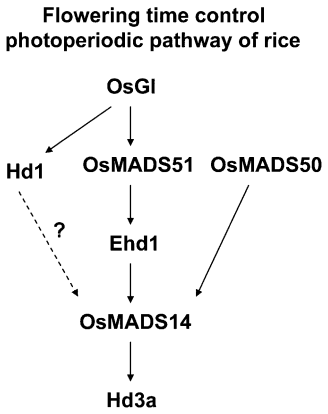

Figure 9. Model of flowering-signal pathways in rice in which photoperiod signal is first translated to *OsGI*, then divided into two independent pathways. One is mediated by *Hd1*; the other by *OsMADS51* and *Ehd1*. *OsMADS50* generates an independent signal that turns on *OsMADS14*. All signals finally activate *Hd3a*, which produces a putative florigen.

et al., 2007). To investigate the relationship of these regulators with *OsMADS51*, we studied their expression patterns in *OsMADS51* mutants. Transcript levels from *OsMADS14* also were examined because this gene is down-regulated in the *Ehd1* mutant background (Fig. 3) and its ectopic expression causes significant early flowering (Fig. 4).

RT-PCR and quantitative real-time PCR analyses demonstrated that *Ehd1* expression is down-regulated in *OsMADS51* mutants but up-regulated in *Ubi*:*OsMADS51* plants. This suggests that *OsMADS51* acts upstream of *Ehd1*. To further explore this relationship between *OsMADS51* and *Ehd1*, we generated *Ehd1* RNAi plants and measured *OsMADS51* mRNA levels in RNAi plants. The previously identified *Ehd1* mutant allele has one amino acid change that reduces DNA-binding ability and reduces Ehd1 function (Doi et al., 2004). Our RNAi results further support the role of *Ehd1* as a flowering activator. In the *Ehd1* RNAi plants, *OsMADS51* mRNA levels are similar between RNAi and control plants, whereas *OsMADS14* and *Hd3a* expression are affected by the mutation. Therefore, combined with the results from our *OsMADS51* mutants, we conclude that *OsMADS51* transmits a flowering signal through *Ehd1*, which then turns on *OsMADS14* and *Hd3a*. *OsMADS51* regulates *Ehd1*, *OsMADS14*, and *Hd3a* not only in SDs but also in LDs. However, the absolute amounts of these flowering activators are quite low in LDs, which is probably not sufficient for inducing flowering signal.

In contrast, levels of *Hd1* and *OsGI* are not notably changed in *OsMADS51* mutants or overexpressing plants, thereby implying that *OsMADS51* acts either downstream of these genes or independently. To dissect these possibilities, we applied antisense technology to evaluate transgenic plants that suppress *OsGI*. Not only the level of *Hd3a*, a known downstream target of *OsGI*, but also the levels of *OsMADS51* and *Ehd1* are down-regulated in the *OsGI* antisense plants. These results suggest that *OsMADS51* acts downstream of *OsGI*. The daily rhythm observed with *OsMADS51* further supports this hypothesis because that rhythm is similar to the one for *OsGI*, showing a peak in the middle of the day (Fig. 7). Therefore, the SD promotion pathway mediated by *OsGI* can be divided into two routes. One is the well-known *Hd1*-mediated pathway, whereas the other is the *OsMADS51*-*Ehd1*-mediated pathway. Our finding is consistent with a previous report that Hd1 works regardless of the function of Ehd1 (Doi et al., 2004). Furthermore, our study confirms that *Hd1* and *Ehd1* operate independently and it provides additional evidence that *OsGI* is a common upstream regulator of *Hd1* and *Ehd1* (Fig. 9).

Both *Ehd1* and *OsMADS51* occur as orphan genes within their family in the rice genome and no putative orthologs have been found in the Arabidopsis genome. Therefore, these unique genes probably function to differentiate SD-inducible rice from LD-inducible Arabidopsis. Thus, we can conclude that *OsMADS51* plays an important role in transmitting the flowering signal from *OsGI* to *Ehd1* in the SD promotion pathway and that this route is independent from previously reported pathways mediated by *Hd1* or *OsMADS50* (Fig. 9).

MATERIALS AND METHODS

Plant Materials and Growth

T-DNA-tagging lines were generated from rice (*Oryza sativa*) var. japonica 'Dongjin' and 'Hwayoung' (Jeon et al., 2000; Jeong et al., 2002). Insertion sites were determined via inverse PCR (An et al., 2003; Ryu et al., 2004; Jeong et al., 2006). T-DNA-tagged mutants were searched for in our flanking sequence database (www.postech.ac.kr/life/pfg/risd), which is also linked to the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/RiceGE; An et al., 2005a, 2005b). Plants were reared in growth rooms under either continuous SDs (10 h light/14 h dark) or LDs (14 h light/10 h dark).

Screening of *OsMADS51* Mutants from T-DNA-Tagging Lines

The *osmads51* homozygous progeny were screened from T_1 seeds of the mutants. Genotyping was performed with 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 40 s, using a combination of specific and T-DNA border primer sets as follows. The primer sets for *osmads51-1* included F2 primer (5′-CAACAACTGACGAGGAGCAC-3′), R2 primer (5′-TAGATCATGACCTCCAGTAC-3′), and Gus2R primer in T-DNA (5′-TTGGGGTTTCTACAGGACGTAAC-3′). The *osmads51-2* primer sets were F3 primer (5′-CCTCCCATGTTGTGTTCCTT-3′), R3 primer (5′-AAATGGCAAACCACTCGAAC-3′), and LB2R primer in T-DNA (5′-ACGTCCGCAATGTGTTATTAAG-3′). The Gus2R and LB2R primers were used to obtain the T-DNA flanking sequences by inverse PCR (An et al., 2003; Jeong et al., 2006).

Vector Construction and Transformation

The full-length cDNA clone of *OsMADS51* (GenBank accession no. AB003327; Shinozuka et al., 1999) was amplified by PCR using a forward primer (5′-aagcttggggagctagggttttcg-3′) and a reverse primer (5′-ggtaccaaactatgcacaggacacc-3′). These primers contained one of two restriction enzyme sites, *Hin*dIII or Asp-718 (underlined in the primer sequences), for subsequent cloning. The PCR product was first cloned into pBluescript SK− (Stratagene).

Afterward, cDNA was subcloned into the pGA1611 binary vector (Lee et al., 1999; Kim et al., 2003) between the maize (*Zea mays*) *Ubi* promoter and the *nopaline synthase* (*nos*) terminator to generate the *Ubi*::*OsMADS51* construct. To create the *Ehd1*-RNAi construct, we PCR amplified the 346-bp *Ehd1* cDNA fragment (724–1,069 bp from the translation initiation site of the *Ehd1* cDNA clone; GenBank accession no.AB092506), using a forward primer (5′-gttgccagtcatctgcag-3′) and a reverse primer (5′-ggatgtggatcatgagacat-3′). The PCR product was first cloned into pGA2960, a Gateway vector modified from pENTR 1A (Invitrogen). Afterward, the product was subcloned into the pANDA (p2K-1) binary vector by a clonase reaction (Miki and Shimamoto, 2004). Our *OsGI* antisense construct was generated by inserting a partial cDNA clone into the binary vector pGA1611 in the antisense orientation. The cDNA clone of *OsGI* (GenBank accession no. AJ133787mRNA) was amplified by PCR using a forward primer (5′-agacagcaaatttgactgcggcag-3′) and a reverse primer (5′-gacatgctgagtgcacggacatgcg-3′). The PCR product was first cloned into a pGEM-T vector (Promega). This *OsGI* cDNA product was then digested with restriction enzymes *Xho*I (991 bp from start codon) and *Xba*I (2,374 bp from start codon). The digested product was cloned into pBluescript SK− and subcloned into the pGA1611 binary vector using *Kpn*I and *Sac*I. Rice transformation was performed according to *Agrobacterium*-mediated cocultivation methods (Lee et al., 1999). All transgenic rice plants were grown in the greenhouse and then transferred to a confined paddy field.

RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated with Tri Reagent (MRC). First-strand cDNA was synthesized from 2 μg of total RNA, using Moloney murine leukemia virus reverse transcriptase (Promega), with 10 ng of the oligo$(dT)_{15}$ primer and 2.5 mM deoxyriboneucleotide triphosphate, as described by Han et al. (2006). *Actin1* was used for normalization of the cDNA quantity. Primers included 5′-GTTTGCTCTGCTCCTACTC-3′ and 5′-ACTCCTCCTCCAGCATTGAA-3′ for *OsMADS51*; 5′-TGGAGAAAGGTTGTGGATGC-3′ and 5′-CATAGACGGCACTTCAGCAGAT-3′ for *OsGI*; 5′-GTTGCCAGTCATCTGCAGAA-3′ and 5′-GGATGTGGATCATGAGACAT-3′ for *Ehd1*; 5′-TTCTCCTCTCCAAAGATTCC-3′ and 5′-CATACGCCTTTCTTGTTTCA-3′ for *Hd1*; 5′-TCCTATGCAGAAAAGGTCCTT-3′ and 5′-GGACGAAGCCAAAATATACAC-3′ for *OsMADS14*; 5′-ATGGCCGGAAGTGGCAGGGAC-3′ and 5′-ATCGATCGGGATCATCGTTAG-3′ for *Hd3a*; and 5′-GTATCCATGAGACTACATACATACAACT-3′ and 5′-TACTCAGCCTTGGCAATCCACA-3′ for *Actin1*. The reactions included an initial 5 min of denaturation at 95°C, followed by 95°C for 15 s, 55°C for 30 s, and 72°C for 40 s. PCR cycles numbered 28 for the flowering-regulator genes and 25 for *Actin1*.

Quantitative Real-Time RT-PCR

Real-time PCR was performed using a Roche LightCycler II as previously described (Han et al., 2006). cDNA was prepared from the leaf blades of plants at 35 or 55 DAG. The *Ubi* mRNA level was used to normalize the expression ratio for each gene. Primers included 5′-AACCAAGATCGGCAGTATGG-3′ and 5′-GATTGATTGCTCCAGCAGGT-3′ for *Hd1*; 5′-GCCAACTAATGCTCGAGTCC-3′ and 5′-CAGCTGCTGCAGGGTAGTTA-3′ for *OsMADS14*; 5′-AGCCCAAGTGACCCTAACCT-3′ and 5′-GTTGTAGAGCTCGGCGAAGT-3′ for *Hd3a*; and 5′-CACGGTTCAACAACATCCAG-3′ and 5′-TGAAGACCCTGACTGGGAAG-3′ for *Ubi*. Changes in gene expression were calculated via the $\Delta\Delta_{Ct}$ method.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB003327, AB092506, and AJ133787.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression profiles for various flowering regulators in 55-DAG *osmads51-2* plants.

Supplemental Figure S2. Expression profiles for various flowering regulators in 35-DAG *Ubi*::*osmads51* plants of line 4.

Supplemental Figure S3. Analyses of relationship between *OsMADS51* and *OsMADS50*.

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