Molecules and Cells

# *Histone Deacetylase 701 (HDT701)* Induces Flowering in Rice by Modulating Expression of *OsIDS1*

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Rice is a facultative short-day (SD) plant in which flowering is induced under SD conditions or by other environmental factors and internal genetic programs. Overexpression of *Histone Deacetylase 701* (*HDT701*) accelerates flowering in hybrid rice. In this study, mutants defective in *HDT701* flowered late under both SD and long-day conditions. Expression levels of florigens *Heading date 3a* (*Hd3a*) and *Rice Flowering Locus T1* (*RFT1*), and their immediate upstream floral activator *Early heading date 1* (*Ehd1*), were significantly decreased in the *hdt701* mutants, indicating that *HDT701* functions upstream of *Ehd1* in controlling flowering time. Transcript levels of *OsINDETERMINATE SPIKELET 1* (*OsIDS1*), an upstream repressor of *Ehd1*, were significantly increased in the mutants while those of *OsGI* and *Hd1* were reduced. Chromatin-immunoprecipitation assays revealed that HDT701 directly binds to the promoter region of *OsIDS1*. These results suggest that HDT701 induces flowering by suppressing *OsIDS1*.

Keywords: flowering time, histone deacetylase, *OsIDS1*, *OsGI*, *Hd1*

## INTRODUCTION

Flowering is one of the most crucial biological processes in plants because it is a prerequisite for the development of fruits and grains. Transition from the vegetative phase is the first step toward reproductive success. Therefore, producing flowers at the appropriate time is a key factor. Whereas early flowering shortens the vegetative phase to an insufficient period that often leads to reduced yields, deferred flowering may also contribute to yield losses when plants in temperate regions are exposed to characteristically colder temperatures later in the growing season. For rice (*Oryza sativa*), chilling at the grain ripening stage results in immature grains while high temperatures are associated with heat damage and a reduction in grain quality. Thus, flowering time is highly correlated with total grain yield and quality in rice (Morita et al., 2017; Sun et al., 2014; Zhang et al., 2015).

The timing of floral transition is regulated by many factors, e.g., internal genetic programming, day length, temperature, nutrient availability, and abiotic/biotic stresses (Cho et al., 2017). In Arabidopsis (*Arabidopsis thaliana*), a long-day (LD) plant, flowering time is accelerated by longer photoperiods. *GIGANTEA* (*GI*) merges signals from photoreceptors and a circadian clock to activate *CONSTANS* (*CO*), which in turn promotes the expression of *Flowering Locus T* (*FT*), a major floral activator that is expressed in the vascular tissues of leaves, all of which lead to the induction of floral transition (Fowler et al.,1999; Park et al., 1999; Samach et al., 2000; Yanovsky and Kay, 2002).

*Oryza sativa GIGANTEA* (*OsGI*), *Heading date 1* (*Hd1*), and *Heading date 3a* (*Hd3a*) are the rice homologues of *GI*, *CO*, and *FT*, respectively. This core flowering pathway is

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conserved in many plant species. Although *CO* enhances flowering in Arabidopsis, *Hd1* has a dual function in rice. Whereas *Hd1* promotes flowering under short-day (SD) conditions by enhancing the expression of *Early heading date 1* (*Ehd1*) (Zhang et al., 2017), the factor suppresses flowering under LD conditions by inhibiting *Ehd1* and *Hd3a* (Hayama et al., 2003). In addition to this conserved flowering pathway, *Flowering Locus C* (*FLC*) in Arabidopsis and *Grain number, plant height, and heading date7* (*Ghd7*) and *Early heading date 1* (*Ehd1*) in rice are unique floral regulators. In these dedicated flowering pathways, *FLC* and *Ghd7* act as major flowering repressors while *Ehd1* functions as a floral activator (Cho et al., 2016; Doi et al., 2004; Sun et al., 2014).

Rice is a facultative SD plant. Its heading date is advanced under SD conditions (<13 h of light/day) but retarded under LD conditions (>14 h of light/day) (Cho et al., 2016; Ishikawa et al., 2011; Kim et al., 2013; Lee et al., 2007; Nishida et al., 2002). Rice has two florigens, *Hd3a* and *Rice Flowering Locus T1* (*RFT1*), that are induced by *Ehd1* (Corbesier et al., 2007; Doi et al., 2004; Ryu et al., 2009). Several transcription factors activate or repress the expression of *Ehd1*, a gene that is a critical convergence point for various flowering signals in rice.

Several genes, including *Ghd7* and *OsMADS56*, preferentially function as suppressors of flowering under LD. However, some constitutive suppressors inhibit flowering regardless of day length. For example, two AP2-like genes, *OsINDETERMINATE SPIKELET 1* (*OsIDS1*) and *SUPERNUMERARY BRACT* (*SNB*), repress the expression of *Ehd1* and florigens, resulting in delayed flowering under both LD and SD conditions. In this pathway, *microRNA172* (*miR172*) degrades transcripts of *OsIDS1* and *SNB* to induce flowering, whereas *Oryza sativa Phytochrome B* (*OsPhyB*) enhances the expression of *OsIDS1* and *SNB* by repressing *miR172* to inhibit flowering (Lee et al., 2014). *OsCOL4*, a member of the CONSTANS-like (COL) family in rice, is up-regulated by *OsPhyB*. The former suppresses flowering under both SD and LD by dampening the transcript levels of *Ehd1* and the florigens via upregulation of floral repressors *OsIDS1* and *SNB* (Lee et al., 2010; 2014). *Oryza sativa LEAFY COTYLEDON 2 and FUSCA 3-LIKE 1* (*OsLFL1*) constitutively deters rice flowering by directly attenuating the transcript level of *Ehd1* (Peng et al., 2007; 2008). Furthermore, *OsLF*, which encodes a typical HLH protein, delays flowering regardless of day length by directly repressing *Hd1* and *OsGI* (Zhao et al., 2011).

The histone acetyltransferases (HATs) and histone deacetylases (HDACs) reversibly catalyze acetylation or deacetylation on histone lysine residues for the transcriptional activation and repression, respectively, of target genes. Plant HDACs can be classified into three major families: the RPD3/HDA1 superfamily, the SIR2 family, and the plant-specific HD2 family (Pandey et al., 2002). In Arabidopsis, histone acetylation and deacetylation are involved in various biological processes such as flowering time, leaf development, seed abortion, and abiotic stress responses (Dangl et al., 2001; He et al., 2003; Luo et al., 2012a; 2015; Sridha and Wu, 2006; Ueno et al., 2007; Wu et al., 2000; 2008). The rice genome contains at least 19 HDAC genes (Hu et al., 2009), including at least two HD2 genes -- *Histone deacetylase 701* (*HDT701*) and *Histone deacetylase 702* (*HDT702*) -- based on phylogenic analysis (Fu et al., 2007). *HDT702* RNAi plants have smaller-diameter stems and much narrower leaves, implying that this gene has a role in cell division or growth (Hu et al., 2009). *HDT701* encodes a histone H4 deacetylase that reduces acetylation levels at the 5th and 16th lysine residues of histone H4. Its overexpression makes rice plants more susceptible to *Magnoporthe oryzae* and *Xanthomonas oryzae* pv. oryzae whereas *HDT701* RNAi plants are resistant to those pathogens. This suggests that *HDT701* functions as a negative regulator in plant innate immunity by modulating histone H4 acetylation of defense-related genes in rice (Ding et al., 2012). Overexpression of *HDT701* also leads to late seed germination due to decreased histone H4 acetylation and reduced expression of GA-biosynthetic genes. In addition, *HDT701*-overexpression transgenic plants display enhanced resistance to salt and osmotic stresses during the seedling stage, thereby denoting the role this gene has in seed germination and responses to abiotic stresses (Zhao et al., 2015). Finally, overexpression of *HDT701* accelerates flowering under natural LD conditions by repressing *OsGI* and *Hd1* (Li et al., 2011).

In this study, we analyzed the role of *HDT701* in determining flowering time by analyzing knockout (KO) mutants. Our results demonstrated that this gene controls flowering time in rice mainly by suppressing *OsIDS1*, which is an upstream suppressor of Ehd1 and florigens.

## MATERIALS AND METHODS

### Plant materials and growth conditions

*Oryza sativa* var. *japonica* cultivars Dongjin and Hwayoung were used to generate the T-DNA tagging lines (Jeon et al., 2000; Jeong et al., 2002). To download the genomic DNA sequences, we accessed the Rice Annotation Project Database (RAP-DB; http://rapdb.dna.affrc.go.jp; Tanaka et al., 2008) and the TIGR Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu; Ouyang et al., 2007). The *hdt701-1* mutant (Line number 1B-05907) was identified from our rice T-DNA insertion sequence database (An et al., 2005a; 2005b; Jeong et al., 2006). Homozygous mutants were confirmed by PCR, using genomic DNA extracted from the leaf blade. The primers for genotyping were TAGCTCCGCCTCCCACCT (F), TGCCCTGGGAGCTGGAATG (R), and AACGCTGATCAATTCCACAG (NGUS1) (Lee et al., 2015). Additional KO alleles of *hdt701* were generated in the ‘Nipponbare’ rice background through CRISPR/Cas9 techniques (Miao et al., 2013). The plants were genotyped by sequencing the CRISPR/Cas9 target region using the genomic DNA extracted from leaf blades. Seeds were germinated either on an MS medium or in soil, as previously described (Yi and An, 2013). Plants were cultured naturally in the paddy field or else in controlled growth rooms maintained under LD conditions (14 h light, 28℃/10 h dark, 22℃; humidity approximately 60%) or SD conditions (12 h light, 28℃/12 h dark, 22℃; humidity approximately 70%), as previously described (Cho et al., 2016).

### RNA isolation and quantitative real-time PCR analyses

Total RNA was isolated from fully grown uppermost healthy leaves with RNAiso Plus (TaKaRa, Japan; http://www.takarabio.com). RNA samples with 260/280 nm ratios of >1.8 (Nano-Drop 2000; Thermo Scientific, Wilmington, DE, USA; http://www.nanodrop.com) were used. First-strand cDNA synthesis was performed with 2 μg of total RNA plus Moloney murine leukemia virus reverse transcriptase (Promega, USA; http://www.promega.com), RNasin® Ribonuclease Inhibitor (Promega), oligo (dT) 18 primer, and dNTP. Afterward, synthesized cDNAs and SYBR Green I Prime Q-Master mix (GENETBIO, Daejeon, Republic of Korea) were utilized to monitor gene expression via quantitative real-time (qRT)-PCR on a Rotor-Gene Q system (QIAGEN, Germany) (Cho et al., 2016; Ryu et al., 2009). Rice *Ubi* was used for normalization. All experiments were conducted at least three times and, for each experiment, more than three independent samples were used. To ensure primer specificity, we performed these experiments only when the melting curve displayed a single sharp peak. The $\Delta\Delta$CT method was applied to calculate changes in relative expression. All primers for quantitative real-time PCR are listed in Supplementary Table S1.

### Vector construction and plant transformation

For constructing the CRISPR/Cas9 vector, the rational CRISPR/Cas9 target sequences with protospacer adjacent motifs were screened with the aid of the CRISPRdirect web server (http://crispr.dbcls.jp; Naito et al., 2015) to find potential target sequences with minimal off-target cleavage. A spacer sequence (AAAGATCATTCCAGCTCCCA) was cloned into entry vector pOs-sgRNA for monitoring the expression of sgRNA. The resulting recombinant entry vector, pOs-sgRNA, was further cloned into a destination vector, pH-Ubi-cas9-7, using the GatewayTM system (Miao et al., 2013). For generation of the HA tag transgenic plants, a full-length cDNA of *HDT701* was isolated by PCR, using two primers: 5'-AAGCTTTAGCTCCGCCTCCCACCT-3' and 5'-ACTAGTCTTGGCGGGGTGCTTGGC-3'. Amplified PCR product was digested with restriction enzymes *Hind*III and *Spe*I, and inserted into the binary vector pGA3428 under the control of the maize *ubiquitin 1* promoter (Kim et al., 2009). The construct was introduced into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method (An et al., 1989). Rice transformation via *Agrobacterium*-mediated co-cultivation was performed as previously reported (Jeon et al., 2000).

### Histochemical assay of GUS activity

The plants were grown for 6 d in MS media under continuous light. After vacuum-infiltration for 30 min, samples were kept overnight at 37℃ in a GUS-staining solution containing 100 mM sodium phosphate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5% Triton X-100, 10 mM EDTA (pH 8.0), 0.1% X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid/cyclohexylammonium salt), 2% DMSO, and 5% methanol (Yoon et al., 2014). Chlorophylls were removed by sequentially incubating the samples in 70% and 95% ethanol at 60℃. The GUS-stained samples were then soaked for 30 to 60 min at room temperature in VISKOL clearing reagent (Phytosys LLC, USA, http://visikol.com/). After resin-sectioning (10 μm thick), GUS activity was visualized with a BX61 microscope (Olympus, Japan).

### *Chromatin-immunoprecipitation* (ChIP) analysis

Transgenic plants expressing HDT701-HA were used for ChIP analysis as previously reported (Yoon et al., 2017). Briefly, 2 g of leaf blade sample were incubated in 3% formaldehyde. After nuclei isolation, chromatins were fragmented to approximately 500- to 1,000-bp lengths by sonication. As an input, 1% of the sample was gathered before pre-clearing. Anti-HA monoclonal antibodies (#2367; Cell Signaling) were used for immunoprecipitation. Data were normalized according to the percent-of-input method (Haring et al., 2007). Tested areas for *OsIDS1* were P1; -1,886 ~ -1,766 bp, P2; -1,633 ~ -1,484 bp, P3; -1,139 ~ -1,265 bp, P4; -953 ~ -808 bp, and P5; -252 ~ -143 bp upstream from the translation start codon ATG. Those for *SNB* were P1; -1,893 ~ -1,766 bp, P2; -1,725 ~ -1,613 bp, P3; -1,517 ~ -1,412 bp, P4; -1,108 ~ -978 bp, P5; -821 ~ -692 bp, and P6; -555 ~ -425 bp upstream from the start codon. The PCR primers for ChIP are listed in Supplementary Table S1. All assays were conducted at least three times, each involving three biological replicates.

## RESULTS

### Identification of late-flowering mutants

A late-flowering mutant line, 1B-05907, was identified by screening T-DNA insertion tagging lines in the paddy field. The T-DNA was inserted in the first intron of *HDT701* (Fig. 1A) and the transcript level for that gene was markedly decreased in the mutant (Fig. 1B). That line displayed a phenotype of flowering that was delayed by about two weeks in the field (Fig. 1C). Because flowering time is regulated by multiple pathways, including day length-preferential routes, we studied the mutant phenotypes under controlled SD and LD conditions. When compared with wild type (WT) controls, flowering of *hdt701-1* mutant plants was delayed by approximately two weeks under SD and three weeks under LD conditions (Fig. 1D). This demonstrated that HDT701 is a constitutive activator of flowering regardless of day length.

In the T-DNA tagging line, the *GUS* coding region was inserted into *HDT701* at the same orientation as the tagged gene. GUS analysis of that line showed a positive response, indicating that *HDT701* was translationally fused to *GUS*. We have previously reported that a translational fusion between a tagged gene and *GUS* can be made even when T-DNA is inserted within an intron (Wei et al., 2017). Analysis of the genomic DNA of the line revealed that only one copy of T-DNA was present in the entire genome, suggesting that GUS expression was likely due to a fusion between HDT701 and GUS. Histochemical GUS analysis of leaf blades showed that GUS signals were ubiquitous in the leaves, including phloem parenchyma cells and mesophyll cells (Fig. 1E). This result is consistent with a previous report that *HDT701* is expressed in various organs (Zhao et al., 2015).

To confirm whether the delay in flowering time was indeed due to a mutation in *HDT701*, we generated additional alleles by the CRISPR/Cas9 method, designing a target site in

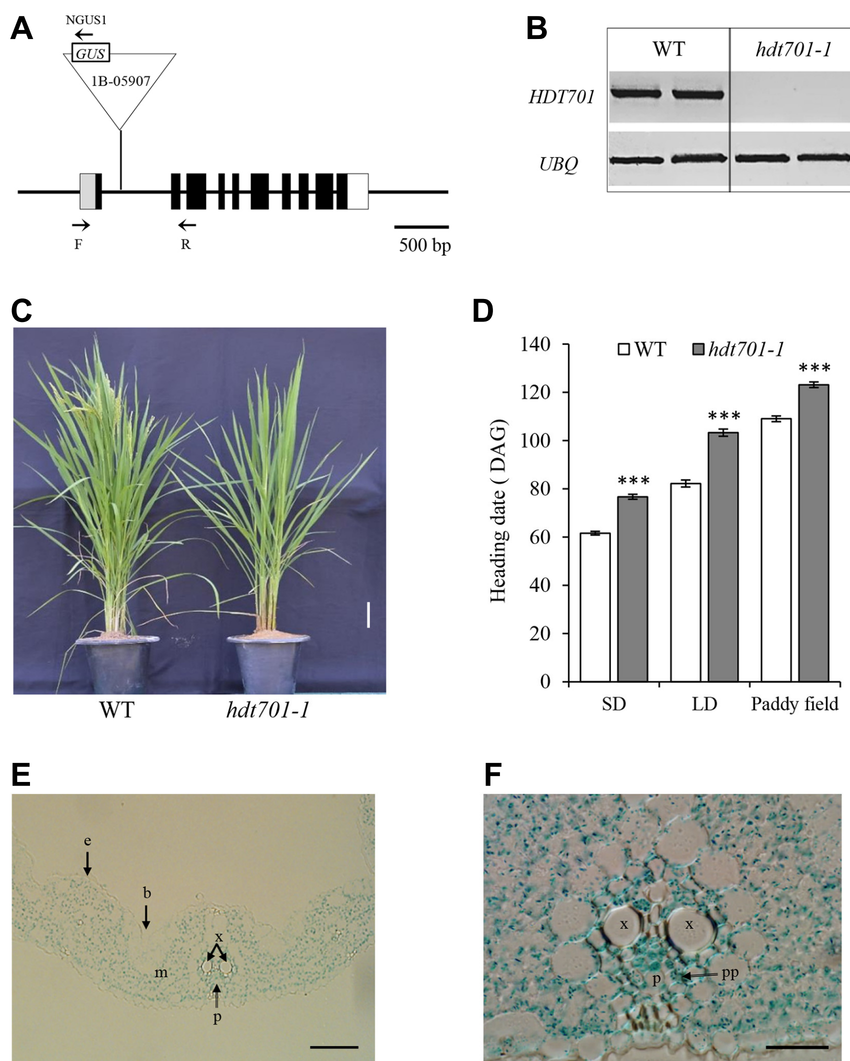


**Fig. 1. Schematic diagram of *HDT701* structure and comparison of flowering times between WT and *hdt701-1* mutants.** (A) Gene structure of *HDT701*. Black boxes indicate exons in coding region; lines connecting boxes, introns; gray box, 5'-UTR region; open box, 3'-UTR region. T-DNA is inserted into first intron of *HDT701* in Line 1B-05907. Direction of promoterless *GUS* reporter gene is indicated within T-DNA (triangle). Primers F, R, and NGUS1 (marked with arrows) were used for genotyping. Scale bar, 500 bp. (B) *HDT701* transcript levels in WT and *hdt701-1* measured by RT-PCR. (C) Phenotypes of *hdt701-1* and WT at heading stage under paddy field conditions. Scale bar, 10 cm. (D) Days to heading for WT and *hdt701-1* plants under SD, LD, and field conditions. DAG, days after germination. Error bar indicate standard deviation; $n = 8$. Levels of significant difference are indicated by *** ($P < 0.005$). (E) GUS-staining of leaf blade cross-section from Line 1B-05907. (F) Close-up of leaf section at vasculature region. b, bulliform cells; e, epidermis; m, mesophyll cells; p, phloem; pp. phloem parenchyma; x, xylem. Scale bars, 50 μm (E) and 20 μm (F).

the 5$^{th}$ exon of *HDT701* (Fig. 2A) and obtaining five independent transgenic lines. Sequencing the flanking regions of that site revealed that CRISPR line #4 had deletions in both chromosomes and line #5 had a single-bp insertion, whereas line #1 did not carry any mutation. Further analyses of the two null mutant lines in the next generations (line #4 and #5) showed late flowering when compared to WT controls. While line #1 which has no mutation flowered at the same time as the WT (Figs. 2B and 2C). These experiments confirmed that defects in *HDT701* delay flowering.

## Expression levels for floral regulators

To elucidate the functional roles of *HDT701* in controlling flowering time, we monitored expression levels of previously identified genes that play critical roles in that event. We studied the effects of *hdt701* mutations under both SD and LD conditions because some regulatory factors function differently depending upon day length. For example, *osgi* mutants display a significant delay in flowering under SD but only a slight delay under LD, indicating that *OsGI* controls flowering time preferentially under SD (Lee and An, 2015).

An earlier study showed that overexpression of *HDT701* represses the expression of *OsGI* and *Hd1*, and induces flowering under natural LD conditions (Li et al., 2011). To verify that expression of these genes was also affected in our KO mutants, we performed qRT-PCR experiments with plants grown under controlled LD conditions. Expression was examined at 49 days after germination (DAG) because florigens and most upstream regulatory genes are active at that time when plants are grown under LD (Lee et al., 2016). Leaves were sampled nine times (2- to 4-h intervals) the day to observe any diurnal patterns. We first analyzed *HDT701* and confirmed that the gene was completely silent throughout the 24-h period in the *hdt701-1* mutant (Fig. 3A and Supplementary Fig. S1A). In the WT, the gene was expressed at higher levels in the dark but at reduced levels under illuminated conditions. This diurnal pattern of expression is similar to that previously reported (Li et al., 2011). Expression of *Hd3a* and *RFT1* was significantly lower in the leaves from mutant plants, indicating that the delay in flowering

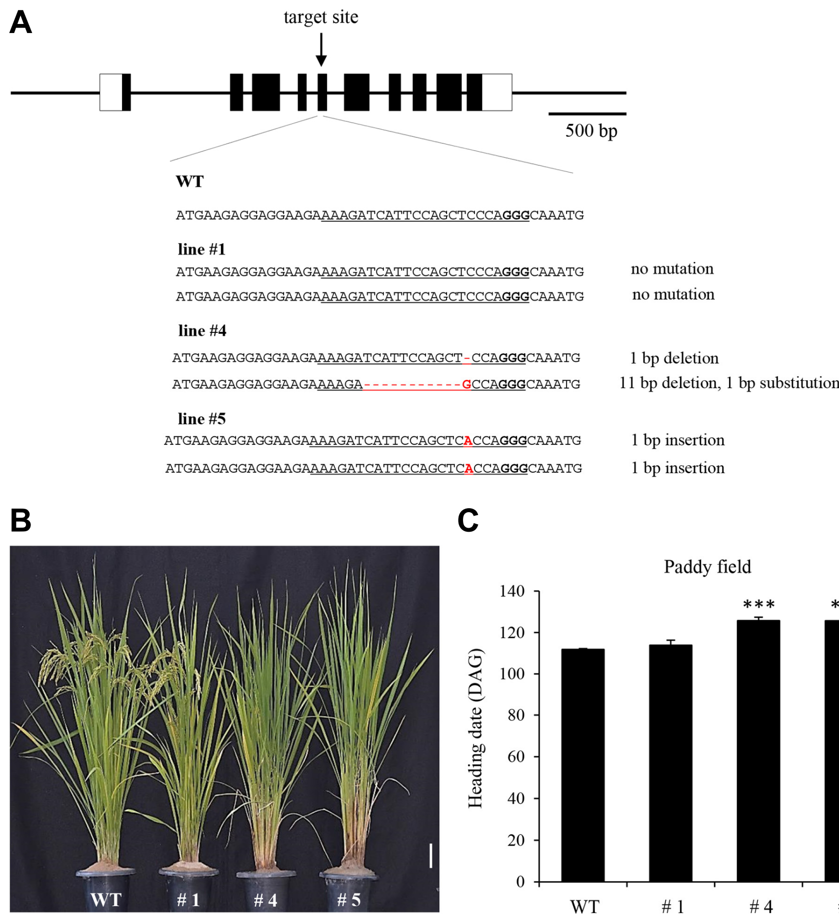


**Fig. 2. Generation of additional *hdt701* alleles by CRISPR/Cas9 method.** (A) Schematic diagram of *HDT701* structure and sequence alignment of sgRNA target region displaying altered bases in mutant lines. The target sequence is underlined. Altered DNA sequences are indicated in red. (B) Phenotypes of *hdt701* KO lines at heading stage. Scale bar, 10 cm. (C) Days to heading for WT, and KO Lines #1, 4, and 5 under natural paddy field conditions. Days to heading was scored when first panicle bolted. Error bars indicate standard deviations; $n$ = 10. Levels of significant difference are indicated by ** ($P < 0.01$) and *** ($P < 0.005$).

was due to reduced expression of the florigens (Figs. 3B and 3C; Supplementary Figs. S1B and S1C). *Ehd1*, an immediate upstream regulator of those genes, was also significantly affected by the mutation (Fig. 3D). Activities of *OsGI* and *Hd1* were slightly decreased in the *hdt701* mutant, especially during the dark period (Figs. 3E and 3F; Supplementary Figs. S1E and S1F). We had not expected to make these observations because overexpression of *hdt701-1* in 'YS63' hybrid rice also reduces the expression of *OsGI* and *Hd1* (Li et al., 2011). If these genes were the main regulatory elements contributing to the flowering phenotype in the *hdt701* mutant, then the KO mutants should have flowered early because OsGI functions upstream of Hd1, a floral repressor under LD conditions. Therefore, the *OsGI-Hd1* pathway does not seem to be the main downstream route from *hdt701-1* to the florigens. Because the *hdt701* mutants flowered late under both SD and LD, the HDT701 target gene is likely a constitutive repressor that functions upstream of *Ehd1*. We previously determined that two AP2 family genes, *OsIDS1* and *SNB*, are constitutive flowering repressors (Lee et al., 2014). Here, expression levels of the former were significantly increased in the mutant (Figs. 3G and Supplementary S1G) while those of the latter were not affected by the mutations (Figs. 3H and Supplementary Fig. S1H). These results suggested that *OsIDS1* is downstream of HDT701. Expression levels of other constitutive repressors, i.e., *OsLFL1*, *OsLF*, and *OsPhyB*, were not altered in the mutant (Figs. 3J-3L; Supplementary Figs. S1J-S1L).

Because flowering by *hdt701* mutants was also delayed under SD, we measured the expression levels of regulatory genes from plants grown under SD conditions. Mature leaf blades were sampled at 28 DAG, when the florigens started to be expressed in SD-grown plants. As we had observed from the LD-grown plants, the mutants expressed no detectable levels of *HDT701* transcript (Figs. 4A and Supplementary Fig. S2A). Expression of the florigens and *Ehd1* was significantly lower in the mutant leaves than in the WT leaves (Figs. 4B-4D; Supplementary Figs. S2B-S2D). Transcript levels of *OsGI* and *Hd1* were also reduced in the mutants, as noted from LD-grown plants, and especially so under SD (Figs. 4E and 4F; Supplementary Figs. S2E and S2F). Because both *OsGI* and *Hd1* function as positive regulatory elements under SD conditions, their decreased expression should have caused late flowering, consistent with the mutant phenotype. Transcript levels of *OsIDS1* were reduced at all nine sampling times, as observed under LD conditions (Figs. 4G; Supplementary Fig. S2G). These results suggested that *OsIDS1* is an important regulator that functions downstream of *HDT701*. Expression was not altered for the other constitutive repressors -- *SNB1*, *OsCOL4*, *OsLFL1*, *OsLF*, and

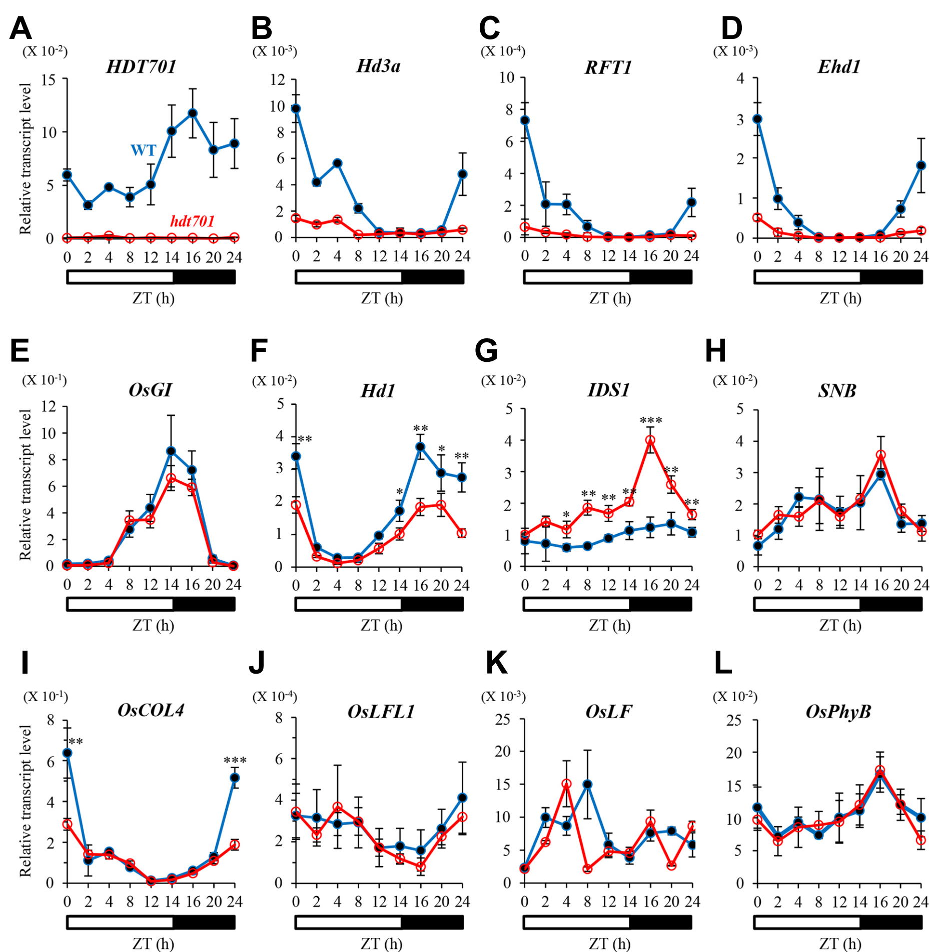


Fig. 3. Diurnal expression patterns of floral regulators in leaf blades of WT and *hdt701-1* plants at 49 DAG under LD. Quantitative RT-PCR analyses of *HDT701* (A), *Hd3a* (B), *RFT1* (C), *Ehd1* (D), *OsGI* (E), *Hd1* (F), *OsIDS1* (G), *SNB* (H), *OsCOL4* (I), *OsLFL1* (J), *OsLF* (K), and *OsPhyB* (L). Closed circles, WT; open circles, *hdt701-1*. y-axis, relative transcript level of each gene compared with that of rice *Ubi*. Error bars indicate standard deviations; $n = 4$ (technical replicates). Levels of significant difference are indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

*OsPhyB* -- in mutant plants grown under SD (Figs. 4H-4L; Supplementary Figs. S2H-S2L). Although expression of *OsCOL4* was reduced at ZT 0 and 24 h under LD (Fig. 3I), it may not be related to the regulatory pathway mediated by *HDT701* because the gene is a constitutive floral repressor and the *hdt701* mutants flowered late under both SD and LD.

## HDT701 directly regulates the expression of *OsIDS1*

*HDT701* is an active histone H4 deacetylase that suppresses expression of target genes via histone deacetylation (Ding et al., 2012; Li et al., 2011; Zhao et al., 2015). To study how HDT701 might directly regulate *OsIDS1* expression, we performed ChIP assays using transgenic plants that express HA-tagged HDT701 as well as transgenics expressing HA alone as a negative control. Four areas (P1, P2, P3, and P4) in the *OsIDS1* promoter region and one area (P5) in the 5′-untranslated region (UTR) were selected for the binding assay (Fig. 5A). Results from the experiments with anti-HA antibodies showed that P4 was preferentially enriched in the chromatins expressing the HDT701-HA fusion protein when compared with the chromatins from transgenic plants expressing HA tag alone (Fig. 5B). However, chromatin enrichment in P1, P2, P3, and P5 was similar between the two types of transgenic plants.

As a negative control, we performed ChIP assays of *SNB* chromatins because this gene encodes a protein that is highly homologous to OsIDS1. Six areas in the *SNB* promoter region were selected for the analysis using plants expressing HDT701-HA or HA tag alone (Fig. 5A). The chromatin enrichment experiments with HA antibodies demonstrated that all six areas were selected equally in the HDT701-HA and HA plants (Fig. 5C). This implied that the promoter region of *OsIDS1* is a potential target of HDT701.

## Regulatory genes that function upstream of *HDT701*

To identify the regulatory genes that function upstream of *HDT701*, we elucidated its expression patterns in various

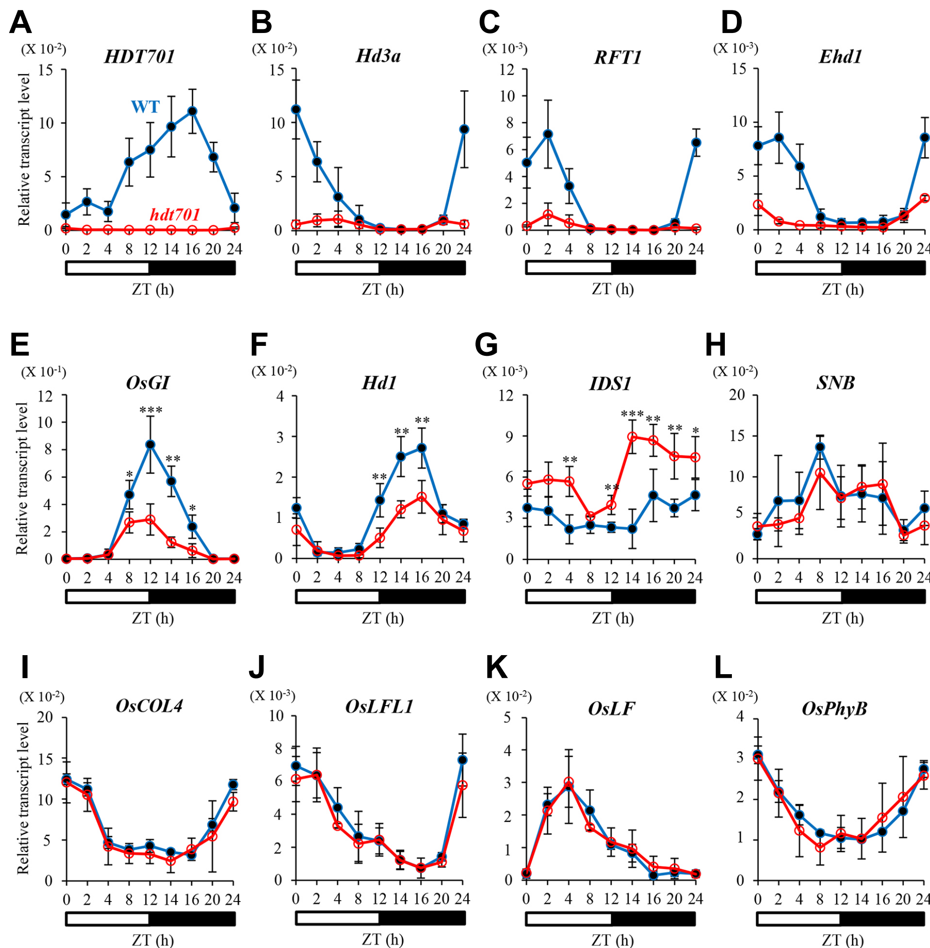


Fig. 4. Diurnal expression patterns of floral regulators in leaf blades of WT and *hdt701-1* plants at 28 DAG under SD. Quantitative RT-PCR analyses of *HDT701* (A), *Hd3a* (B), *RFT1* (C), *Ehd1* (D), *OsGI* (E), *Hd1* (F), *OsIDS1* (G), *SNB* (H), *OsCOL4* (I), *OsLFL1* (J), *OsLF* (K), and *OsPhyB* (L). Closed circles, WT; open circles, *hdt701-1*. y-axis, relative transcript level of each gene compared with that of rice *Ubi*. Error bars indicate standard deviations; $n = 4$ (technical replicates). Levels of significant difference are indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

flowering-time mutants. Transcript levels of *HDT701* were not changed in mutants defective in *OsPhyB* and *OsCOL4*, two positive regulators of *OsIDS1* (Figs. 6A and 6B, Supplementary Figs. S3A and S3B). Likewise, expression was not altered in the *hd1* and *osgi* mutants (Figs. 6C and 6D; Supplementary Figs. S3C and S3D).

## DISCUSSION

We investigated the role of *HDT701* in controlling flowering time using KO mutants generated by T-DNA insertions and CRISPR/Cas9. The mutant plants flowered later than the WT due to reductions in the expression levels of *Hd3a*, *RFT1*, and *Ehd1*. This indicated that *HDT701* is a floral activator that functions upstream of *Ehd1*. Our result is consistent with other observations of *HDT701*-overexpression plants, which flower early because of induced expression of the three genes (Li et al., 2011). The previous experiments were conducted under natural LD conditions (Li et al., 2011). In our current study, we observed that the gene is a constitutive repressor of flowering under both LD and SD. Because *HDT701* encodes histone 4 deacetylase, deacetylation of floral repressors would enhance florigen expression. Several histone deacetylation (HDA) genes also control flowering time in Arabidopsis (He et al., 2003). Constitutive delayed-flowering phenotypes of mutants defective in *HDA5* and *HDA6* under both LD and SD conditions imply that histone deacetylation accelerates flowering time in Arabidopsis, similar to that observed in our study (c.f., Luo et al., 2015; Wu et al., 2008).

Histochemical staining of *hdt701-1* transgenic plants showed that *HDT701* is expressed not only in mesophyll cells but also in phloem parenchyma cells, indicating that the gene has multiple functions. In addition to its role in controlling flowering time, this gene is involved in plant innate immunity, GA biosynthesis, and abiotic stress responses (Ding et al., 2012; Zhao et al., 2015). Florigens as well as upstream regulatory genes such as *Ehd1* and *Ghd7* are preferentially

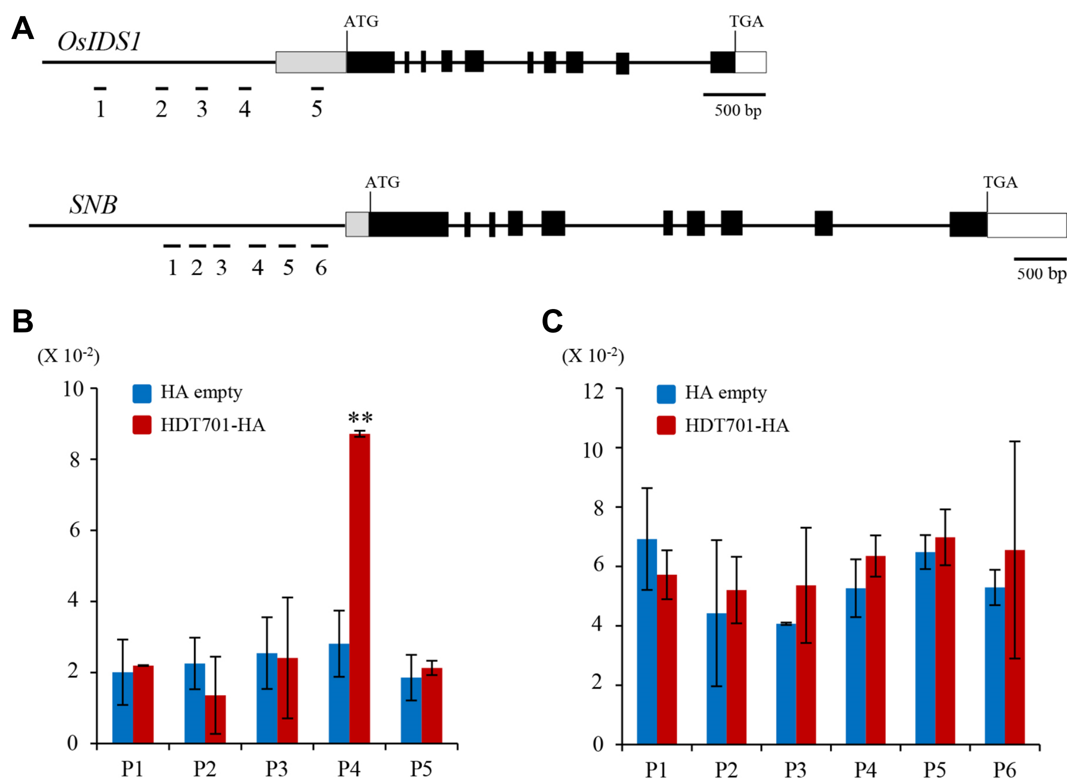


Fig. 5. Chromatin-immunoprecipitation (ChIP) assays of *OsIDS1* chromatin and SNB chromatin. (A) Genomic structures of *OsIDS1* and *SNB*. Tested areas are numbered. (B) ChIP assay of HDT701 enrichment on *OsIDS1* chromatin, using HDT701-HA-tagged transgenic plants. Transgenic plants expressing HA tag alone served as control. Leaf blades were harvested at 30 DAG under SD. Percent-of-input method was used for normalization. (C) ChIP assay of HDT701-HA enrichment on SNB chromatin, as described in (B). Levels of significant difference are indicated by ** ($P < 0.01$). Error bars show standard deviations, $n = 3$. The entire experiment was conducted two times.

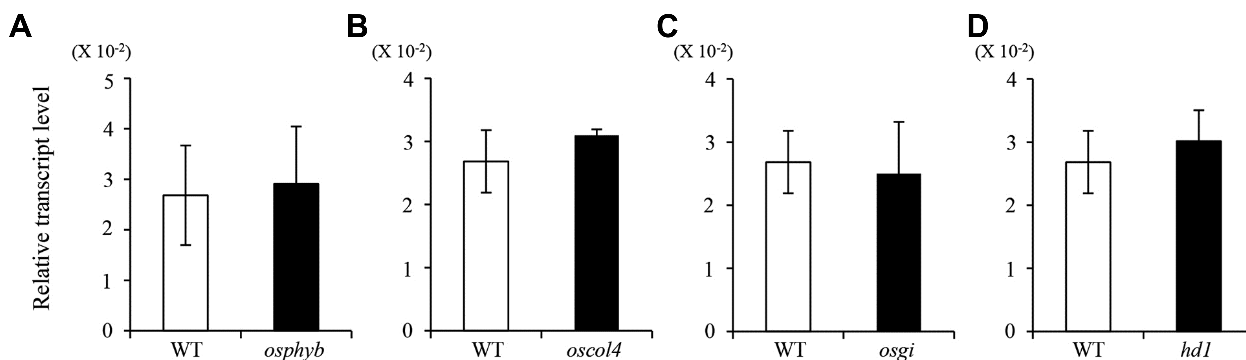


Fig. 6. Expression levels of *HDT701* in *osphyb* (A), *oscol4* (B), *osgi* (C), and *hd1* (D). Total RNAs were prepared from leaf blades at 42 DAG under LD. Error bars display standard deviations; $n = 4$ (technical replicates).

expressed in phloem parenchyma cells, whereas other regulatory genes such as *OsCOL4*, *Hd1*, *OsGI*, and *OsPhyB* are strongly expressed in mesophyll cells (Komiya et al., 2008; Lee et al., 2010; Saito et al., 2012; Tamaki et al., 2007; Xue et al., 2008). Therefore, these findings suggest that HDT701 may function in multiple pathways to influence flowering time.

In a previous study, Li et al. (2011) proposed that *HDT701* induces flowering by suppressing *OsGI* and *Hd1* under LD; this was based on observations that overexpression of the former caused a reduction in expression for the latter two. However, the decline in expression of *OsGI* in the *HDT701*-overexpression plants should have resulted in delayed flowering because OsGI is a flowering enhancer. That research group also reported that transcript levels of *OsGI* and *Hd1* were not altered under SD conditions. We found here that

transcript levels of the two upstream regulatory genes were reduced in *hdt701* KO mutants regardless of day length. This discrepancy might have been due to the cultivar used for generating the transgenic plants. Alternatively, overexpression of the gene may have caused side effects by forming unusual protein complexes.

We identified *OsIDS1* as being downstream of *HDT701* because expression of the former was significantly enhanced under both SD and LD in the *hdt701* mutant. Direct interaction of HDT701 on *OsIDS1* chromatins was indicated by our ChIP assay. OsIDS1 is a member of the AP2 family, which is involved in various processes (Lee et al., 2014). For example, six Arabidopsis members in this family delay flowering and are suppressed by *miR172* (Lee et al., 2014). Similarly, increasing expression of *Zea mays* GROSSY 15, an AP2 member, delays flowering (Zhu and Helliwell, 2011). We previously reported that rice AP2 members OsIDS1 and SNB act as negative regulatory elements in flowering, and their transcripts are targeted by *miR172* (Lee et al., 2014). Although *SNB* is closely related to *OsIDS1*, its transcript levels were not affected in *hdt701* mutants. This suggests that HDT701 specifically selects *OsIDS1* chromatin even though the chromatin-remodeling factor appears to target multiple genes.

In *hdt701* mutants, the mRNA levels of *OsGI* were constitutively down-regulated. That gene plays a positive role in enhancing florigen expression and flowering induction under both LD and SD, although the effect is more severe under SD (Lee et al., 2015). Therefore, the delayed flowering phenotype of the mutant could be explained by lower expression of *OsGI*. However, that reduction in expression was not very significant under LD, although the delay in flowering by *hdt701* mutants was equally significant under both LD and SD.

Transcript levels of *Hd1* were also significantly diminished regardless of day length. Because *OsGI* positively controls the expression of *Hd1* (Hayama et al., 2003), the decrease in expression for *Hd1* could have resulted from the down-regulation of *OsGI* in the mutants. Although Hd1 advances flowering under SD, the regulatory element inhibits flowering under LD. Therefore, the reduction in *Hd1* expression in the *hdt701* mutant under LD would accelerate flowering rather than suppress that process. Therefore, we conclude that the delay in flowering by the mutants under LD was not due to an alteration of the *OsGI* and *Hd1* pathway. It is probable that *HDT701-OsIDS1-Ehd1* is the major pathway under LD. However, under SD, both the *HDT701-OsGI-Hd1-Ehd1* and *HDT701-OsIDS1-Ehd1* pathways appear to modulate florigen expression (Fig. 7).

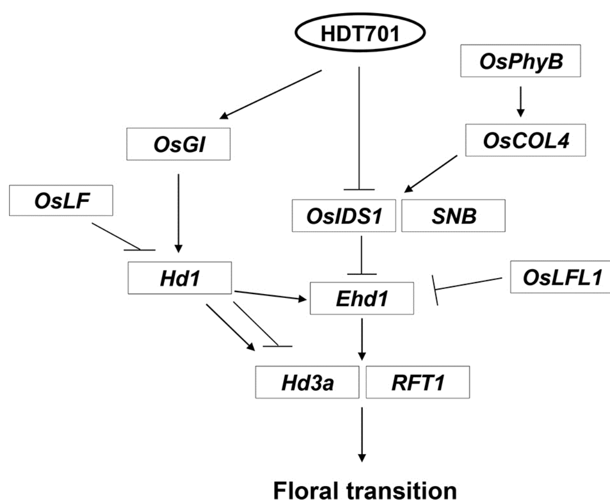

Fig. 7. Model for regulatory pathway governed by *HDT701* for control of flowering time.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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