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RESEARCH ARTICLES

The Histone Methyltransferase SDG724 Mediates H3K36me2/3 Deposition at *MADS50* and *RFT1* and Promotes Flowering in Rice[W][OA]

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Chromatin modifications affect flowering time in the long-day plant *Arabidopsis thaliana*, but the role of histone methylation in flowering time regulation of rice (*Oryza sativa*), a short-day plant, remains to be elucidated. We identified a late-flowering *long vegetative phase1* (*lvp1*) mutant in rice and used map-based cloning to reveal that *lvp1* affects the SET domain group protein 724 (SDG724). SDG724 functions as a histone methyltransferase in vitro and contributes to a major fraction of global histone H3 lysine 36 (H3K36) methylation in vivo. Expression analyses of flowering time genes in wild-type and *lvp1* mutants revealed that *Early heading date1*, but not *Heading date1*, are misregulated in *lvp1* mutants. In addition, the double mutant of *lvp1* with *photoperiod sensitivity5* (*se5*) flowered later than the *se5* single mutant, indicating that *lvp1* delays flowering time irrespective of photoperiod. Chromatin immunoprecipitation assays showed that *lvp1* had reduced levels of H3K36me2/3 at *MADS50* and *RFT1*. This suggests that the divergent functions of paralogs *RFT1* and *Hd3a*, and of *MADS50* and *MADS51*, are in part due to differential H3K36me2/3 deposition, which also correlates with higher expression levels of *MADS50* and *RFT1* in flowering promotion in rice.

INTRODUCTION

Flowering time is affected by both endogenous genetic factors and exogenous environmental signals. In *Arabidopsis thaliana*, a complex genetic network controlling the transition to flowering consists of four pathways, the autonomous, photoperiod, vernalization, and gibberellin pathways (Komeda, 2004). In the short-day (SD) plant rice (*Oryza sativa*), flowering (heading date) is an important agronomic trait for climatic and regional adaption; breeding for this trait allowed rice varieties to be selected for growth at various latitudes, altitudes, and seasons (Izawa, 2007a). Thus, heading date studies are essential for understanding the genetic and biochemical mechanism of flowering and have a significant impact on sustainable agricultural production of rice.

There are at least two independent flowering pathways in rice. The *Heading date1* (*Hd1*) pathway is conserved between rice and *Arabidopsis*, but the *Early heading date1* (*Ehd1*) pathway is unique to rice (Doi et al., 2004). In rice, homologs of *CONSTANS*, *FLOWERING LOCUS T* (*FT*), and *GIGANTEA* (*GI*) are *Hd1*, *Heading date 3a* (*Hd3a*), and Os *GI*, respectively (Yano et al., 2000; Hayama et al., 2002, 2003; Kojima et al., 2002). Although Os *GI* regulates *Hd1*, as in *Arabidopsis*, *Hd1* plays a more enigmatic role in rice. *Hd1* represses flowering under long-day (LD) conditions but promotes flowering slightly under SD conditions, by controlling the expression of *Hd3a* (Hayama et al., 2003; Tamaki et al., 2007; Komiya et al., 2008). *Ehd1*, which encodes a B-type response regulator, is a unique transcriptional regulator and promotes flowering by controlling *FT-like* gene expression independent of *Hd1* under both SD and LD conditions in rice (Doi et al., 2004; Izawa, 2007a). Recently, mounting evidence indicates that *Ehd1* is an important integrator of the floral transition in rice. Several genes belonging to multiple pathways that control the expression level of *Ehd1* have been identified (Komiya et al., 2009). *PHOTOPERIOD SENSITIVITY5* (*SE5*), the rice homolog of *Arabidopsis HEME OXYGENASE1*, encodes a key heme oxygenase involved in phytochrome

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chromophore biosynthesis (Izawa et al., 2000, 2002). *SE5* acts as a floral repressor and delays heading date mainly through suppressing *Ehd1* expression (Izawa, 2007b; Andrés et al., 2009). Rice *MADS50*, a homolog of the *Arabidopsis* gene *SUPPRESSOR OF OVEREXPRESSION OF CO1*, is upstream of *Ehd1* but works either parallel with or downstream of Os *GI* (Lee et al., 2004; Ryu et al., 2009). Rice *MADS51* is a type I MADS box gene that acts downstream of *GI* and upstream of *Ehd1* and promotes flowering transition under SD conditions (Kim et al., 2007). *Ehd2* (also known as *ID1*/*RID1*), which encodes a Cys-2/His-2 zinc-finger transcription factor, is a homolog of maize *Indeterminate1* (*ID1*) and promotes flowering under both SD and LD conditions by upregulating expression of *Ehd1* (Matsubara et al., 2008; Park et al., 2008; Wu et al., 2008). *Grain number, plant height, and heading date7* (*Ghd7*) encodes a CCT domain protein, acts upstream of *Ehd1*, and delays flowering under LD conditions (Xue et al., 2008). Florigen, the mobile signal that moves from an induced leaf to the shoot apex and activates the floral transition at the apical meristem, remained elusive for over 70 years (Knott, 1934) but has now been proposed to be the FT protein in both *Arabidopsis* and rice (Corbesier et al., 2007; Tamaki et al., 2007). In rice, *RICE FLOWERING LOCUS T1* (*RFT1*) and *Hd3a*, which are two homologs of *FT*, were shown to qualify as florigen genes (Komiya et al., 2009; Tsuji et al., 2011). In rice plants with suppressed activity of these two genes, no heading was under either SD or LD conditions (Komiya et al., 2009; Tsuji et al., 2011). Furthermore, many regulators of the *RFT1* florigen, such as *MADS50*, *Ehd1*, *Hd1*, *Ghd7*, and *Ehd2*/*ID1*/*RID1*, form a LD flowering regulatory network in rice (Komiya et al., 2009).

Chromatin structure is important for eukaryotic gene expression, and histone Lys methylation has drawn special attention due to its complex role in this process (Wu et al., 2009; Qiao et al., 2011). Histone Lys methylation in plants functions in biological processes such as flowering transition, floral organ development, carotenoid biosynthesis, shoot and root branching, pollen and macro-trichome development, and the brassinosteroid signaling pathway (Kim et al., 2005; Zhao et al., 2005; Y. Ding et al., 2007; Dong et al., 2008; Xu et al., 2008; Cazzonelli et al., 2009a, 2009b; Grini et al., 2009; Berr et al., 2011; Deal and Henikoff, 2011; Feng and Jacobsen, 2011; Thorstensen et al., 2011; Sui et al., 2012). In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*) and other flowering time genes are regulated by different kinds of histone Lys methylations (Michaels and Amasino, 1999; He and Amasino, 2005; He, 2009; Berr et al., 2011; Deal and Henikoff, 2011; Feng and Jacobsen, 2011; Thorstensen et al., 2011). SET domain proteins, named after Su(var)3-9, E (z), and Trithorax (TRX), are bona fide histone Lys methyltransferases (HMTase) that modify specific histone Lys residues (Ng et al., 2007; Thorstensen et al., 2011). Histone H3 lysine 36 (H3K36) methylation is likely linked to transcription elongation, and H3K36 HMTase genes are involved in regulating the floral transition of *Arabidopsis* (Thorstensen et al., 2011). For example, lesions in *SET DOMAIN GENE8* (*SDG8*) cause early flowering due to a decrease in H3K4 and H3K36 methylation levels at the *FLC* locus (Kim et al., 2005; Zhao et al., 2005; Ko et al., 2010), and lesions in *SDG25*/*ARABIDOPSIS TRITHORAX-RELATED7* also promote flowering due to a suppression of *FLC* expression (Berr et al., 2009; Tamada et al., 2009).

Currently, there is little evidence that chromatin modification participates in the flowering transition of rice. Previously, we characterized *SDG714*, a histone H3K9 HMTase coding gene that did not affect flowering time in rice (Y. Ding et al., 2007). Here, we report that a major HMTase-encoding gene, *Long vegetative phase 1* (*LVP1*)/*SDG724*, is required for H3K36 methylation and promotes heading date in rice. The loss-of-function mutant *lvp1* has a late flowering phenotype under both LD and SD conditions, associated with the suppressed expression of *MADS50*, *MADS51*, *Ehd1*, *RFT1*, and *Hd3a*. Furthermore, our results suggest a novel mechanism for the epigenetic regulation of flowering in rice, in which SDG724 mediates H3K36me2/3 deposition at the *MADS50* and *RFT1* loci and promotes flowering through *MADS50*/*MADS51*-*Ehd1*-*Hd3a*/*RFT1* pathways.

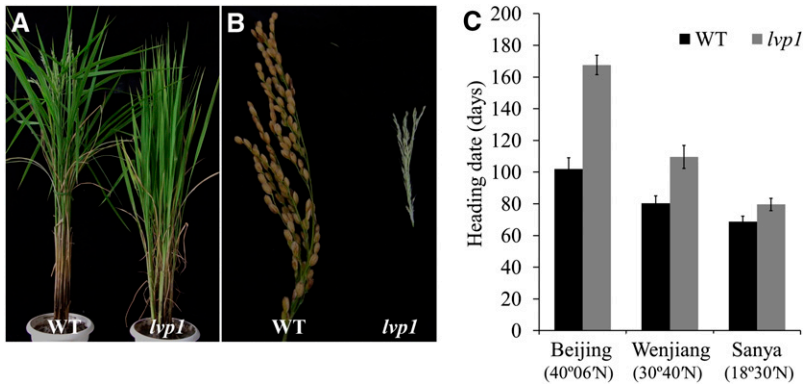

Figure 1. Phenotype of the *lvp1* Mutant.

(A) Phenotypes of 110-d-old wild-type (WT; left) and *lvp1* (right) mutant plants grown in a paddy field at Beijing.

(B) Panicles of 150-d-old wild-type (left) and *lvp1* plants (right) plants grown in Beijing. The *lvp1* mutant was not heading at this time and only developed immature panicles.

(C) Heading date investigation of wild-type and *lvp1* plants grown at three places of different latitudes.

Error bars indicate SD (n = 20).

RESULTS

The *lvp1* Mutant Has a Daylength-Independent Long Vegetative Phase

We previously generated a large population of T-DNA rice mutants in the Nipponbare cv background (Ma et al., 2009), which we screened under natural LD conditions for flowering time mutants in Beijing, China. One of the late-heading mutants identified was named *long vegetative phase1* (*lvp1*; referred to as *lvp1-1*). The phenotype of *lvp1* did not cosegregate with the T-DNA insertion, and an *lvp1* plant without the T-DNA insertion was isolated for further analysis. Under Beijing field conditions, *lvp1* plants did not show heading even in November, 160 d after germination, when the weather became too cold for rice growth (Figure 1). Wild-type and *lvp1* plants were then grown in three locations with different latitudes, Sanya (18°30′N, 110°01′E, SD), Wenjiang (30°40′N, 103°51′E, LD), and Beijing (40°06′N, 116°24′E, LD). The rice growing season in Sanya coincided with a natural SD photoperiod, the one in Beijing with a natural LD photoperiod, and the one in Wenjiang with an intermediate photoperiod between Sanya and Beijing. We surveyed the heading dates of wild-type and *lvp1* plants in all three locations and found that the heading dates of *lvp1* were delayed in all three locations compared with the wild type: from 102 to 168 d in Beijing, from 80 to 110 d in Wenjiang, and from 69 to 80 d in Sanya (Figure 1C). We also grew *lvp1* mutant and wild-type plants under SD (10 h light/14 h dark) and LD conditions (14 h light/10 h dark) in growth chambers. Under SD conditions, the flowering time of the *lvp1* mutant was 76 d, 12 d later than for wild-type plants (64 d; see Supplemental Figure 1 online). Under LD conditions, the *lvp1* mutant did not flower after more than 200 d, whereas wild-type plants flowered after 82 d (see Supplemental Figure 1 online). These data suggest that the *LVP1* gene plays an important role in the floral transition under both LD and SD conditions in rice. However, the difference in

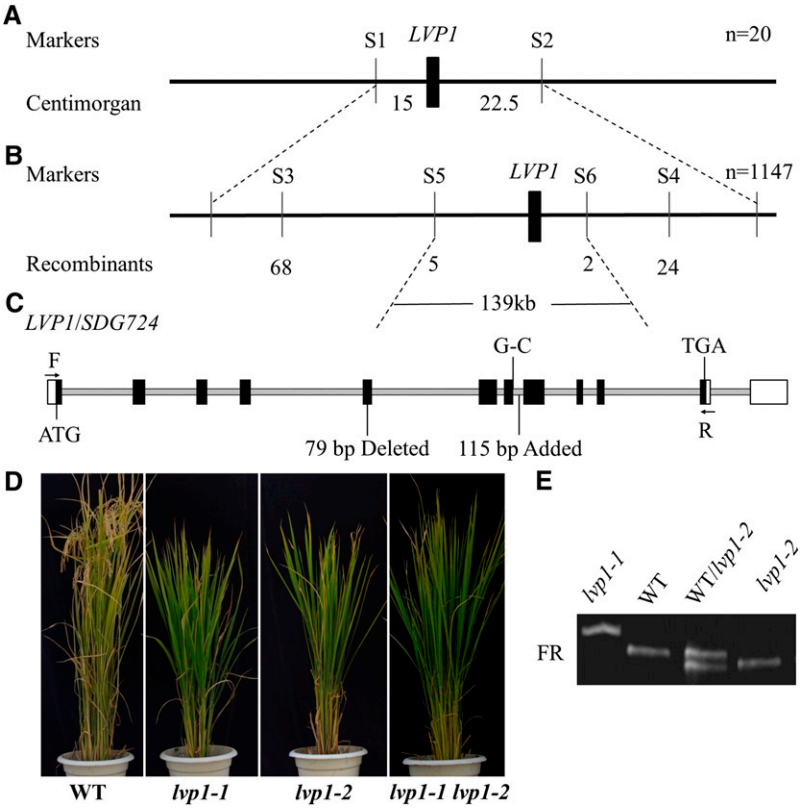

Figure 2. Map-Based Cloning of *LVP1*.

(A) Primary mapping of *LVP1* localized the gene to the region between the molecular markers S1 and S2 on chromosome 9.
(B) Fine mapping of *LVP1* using 1147 F2 progeny plants further delimited the gene to a 139-kb genomic region on chromosome 9.
(C) Genomic structure of *LVP1/SDG724*. Black boxes show exons, white boxes show untranslated regions, and brown lines show introns. "G-C" and "115 bp Added" describe the mutation of the *lvp1-1* allele; "79 bp Deleted" describes the mutation of the *lvp1-2* allele. F and R indicate the primer pair used in **(E)** and are listed in Supplemental Table 1 online.
(D) Allelism test between *lvp1-1* and *lvp1-2*. WT, wild type.
(E) Genotyping of *SDG724* transcripts in wild-type, *lvp1-1*, and *lvp1-2* plants by RT-PCR. The FR primer pairs are indicated in **(C)** and listed in Supplemental Table 1 online.

flowering time between wild-type and *lvp1* plants was smaller under SD conditions, although the mutation delayed flowering time dramatically under LD conditions.

Map-Based Cloning of *LVP1*

Genetic analysis demonstrated that the late flowering phenotype of *lvp1* segregated as a complete monogenic recessive trait. Therefore, we carefully selected 1147 extremely late-heading plants from an F2 population derived from a cross between *lvp1* and Minghui 63 and used a map-based cloning strategy to identify the candidate gene. Using simple sequence repeat and sequence-tagged site (STS) markers, we initially mapped the candidate gene to a 6-Mb genomic region between markers S1 and S2 on chromosome 9 (Figure 2A) and then narrowed down the gene to an ~139-kb genomic region (Figure 2B). According to a rice annotation project database (http://rapdb.dna.affrc.go.jp/), there are 16 putative genes in this region, coding for three hypothetical proteins, 11 retrotransposon proteins, one SET domain–containing protein, and one zinc-finger protein. RT-PCR results showed that the transcribed fragment of the *SDG Os09g0307800* (*SDG724*) was longer in the *lvp1-1* mutant than in wild-type plants (Figure 2E). Sequencing results revealed that an AG-to-AC substitution occurred at the splice site between the seventh intron and exon of *SDG724* and that the entire 115 bp of the seventh intron was retained in *lvp1-1* derived transcripts, leading to a premature stop codon in the transcript of the *lvp1-1* allele (Figure 2C). The AG/AC substitution in *SDG724* was confirmed in genomic DNA of *lvp1* mutant plants.

Further screening of our mutant rice population yielded a second allele, *lvp1-2*, in which the fifth exon of the *SDG724* transcript was deleted (Figures 2C and 2E). The *lvp1-2* allele flowered as late as *lvp1-1* plants under both SD and LD conditions. Crosses between *lvp1-1* and *lvp1-2* mutants produced late flowering in both in F1 and F2 populations, confirming that the two mutants are allelic (Figure 2D).

To confirm that the lesions in *SDG724* were responsible for the late-flowering phenotype of *lvp1* mutants, a genomic fragment containing the entire 6631-bp *SDG724* coding sequence and 1963-bp 5′-upstream region and 1346-bp 3′-downstream noncoding region was obtained from the BAC clone AP006235 and transformed into *lvp1-1* mutant plants. We obtained 30 transgenic lines, as confirmed by PCR. Heading date analyses showed that the flowering time defect was rescued in the transgenic plant lines (see Supplemental Figure 2 online). Taken together, these results confirmed that lesions in *SDG724* were responsible for the late-flowering phenotype of *lvp1* plants.

SDG724 Encodes a SET Domain–Containing Protein

SET domain–containing proteins are well annotated and characterized in *Arabidopsis* (Springer et al., 2003; He, 2009; Liu et al., 2010; Berr et al., 2011; Deal and Henikoff, 2011; Feng and Jacobsen, 2011; Thorstensen et al., 2011). In the rice genome, at least 35 SET protein coding genes were annotated (Ng et al., 2007), but few have been characterized (Liang et al., 2003; Thakur et al., 2003; B. Ding et al., 2007; Y. Ding et al., 2007; Qin et al., 2010; Sui et al., 2012). The plant SET domain–containing

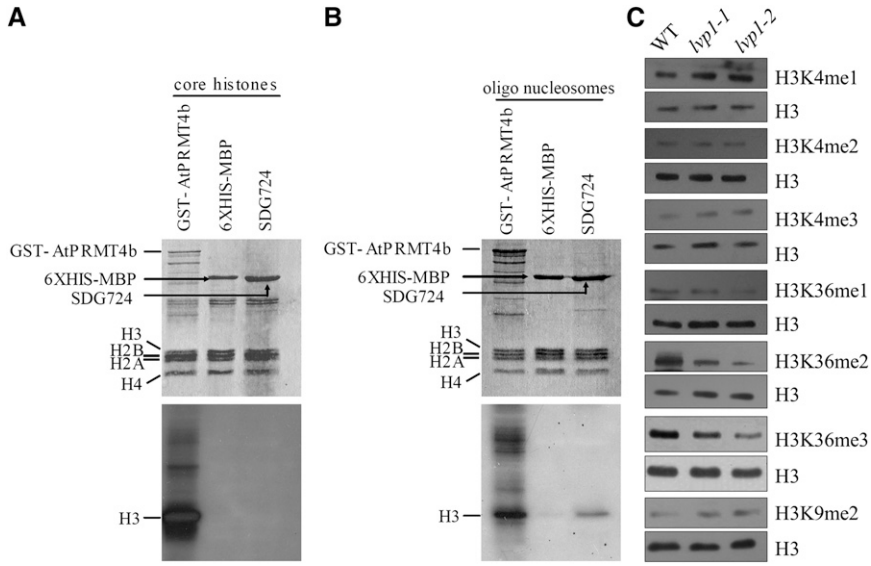

Figure 3. SDG724 Acts as a SET Domain–Containing Histone Methyltransferase.

(A) and **(B)** In vitro HMTase assay analyses. Calf thymus core histones **(A)** and oligonucleosomes **(B)** were used as substrates, and tritium-labeled SAM was used as methyl donor. Proteins analyzed for enzyme activity are pointed out with arrows. Top panel: Coomassie blue–stained protein gel. Bottom panel: Corresponding autoradiograph.

(C) Analysis of the global levels of H3K36me1/2/3, H3K4me1/2/3, and H3K9me2 markers in wild-type (WT), *lvp1-1*, and *lvp1-2* plants using H3K methylation tag-specific antibodies. Histone-enriched proteins were extracted from leaves of 50-d-old plants grown in Beijing under natural LD conditions. For each immunoblot, the same membrane was stripped and reblotted with an antibody against H3 as a loading control.

proteins have been divided into seven classes. SDG724 is considered a class II SET domain protein. Class II members possess three conserved domains, an AWS (for Associated with SET) domain, a SET domain, and a Cys-rich post-SET domain (Ng et al., 2007). Rice has four class II *SDG* genes: *SDG708*, *SDG724*, *SDG725*, and *SDG736* (Ng et al., 2007). As a distinguishing feature, *SDG724* has a more centrally located SET domain (Ng et al., 2007), which may suggest a unique role for *SDG724* in rice. We performed a phylogenetic analysis of class II SDGs using *Arabidopsis*, rice, and maize (*Zea mays*) protein sequences (see Supplemental Data Set 1 online). This analysis indicated that SDG110 from maize and SDG7 and SDG24 from *Arabidopsis* are the closest homologs of SDG724 (see Supplemental Figure 3A online). Therefore, further protein sequence alignment analysis was done among these four SDG members. The result showed that SDG724 shared the highest sequence similarity with SDG110 in maize and differed from the *Arabidopsis* proteins SDG7 and SDG24 by a 20–amino acid deletion in its C-terminal region, which may suggest functional divergence of the SDGs between monocotyledonous and dicotyledonous plants (see Supplemental Figure 3B online).

SDG724 Methylates Oligonucleosomes in Vitro

Several plant SDGs have in vitro HMTase activities (Liu et al., 2010; Berr et al., 2011; Deal and Henikoff, 2011; Thorstensen et al., 2011). For instance, SDG714 in rice tends to choose core histones as preferred substrates (Y. Ding et al., 2007), but *Arabidopsis* SDG8, SDG26, and SDG25 prefer to methylate oligonucleosomes (Xu et al., 2008; Berr et al., 2009). To test in vitro enzymatic activity of SDG724, recombinant protein was expressed in *Escherichia coli* and purified. After removal of the purification tags, the native SDG724 protein was used in methyltransferase activity assays using tritium-labeled *S*-[methyl-3H]-adenosyl-L-Met (SAM) as the methyl donor and core histones and oligonucleosomes as substrates. Compared with the positive control, *Arabidopsis* protein Arg methyltransferase 4b (PRMT4b; Niu et al., 2008), SDG724 showed little enzymatic activity when core histones were used as substrates (Figure 3A). However, when the substrates were changed to oligonucleosomes, a signal was observed at the position corresponding to histone H3 in the SDG724 reaction system (Figure 3B). Thus, SDG724 preferred oligonucleosomes rather than core histones as a substrate and acted as a histone H3 methyltransferase in vitro.

SDG724 Is a Major HMTase Responsible for H3K36 Methylation

In *Arabidopsis*, certain HMTases act as major factors that control specific histone methylation of the entire genome (Liu et al., 2010; Berr et al., 2011; Deal and Henikoff, 2011; Thorstensen et al., 2011). For example, SDG8 is the major in vivo H3K36 HMTase required for global H3K36me2/3 modifications (Zhao et al., 2005; Xu et al., 2008). To investigate whether SDG724 also had a global effect on specific histone H3 sites, immunoblot analyses using a series of antibodies that recognize specific histone Lys methylation modifications were done to identify histone modification differences between *lvp1* mutant and wild-

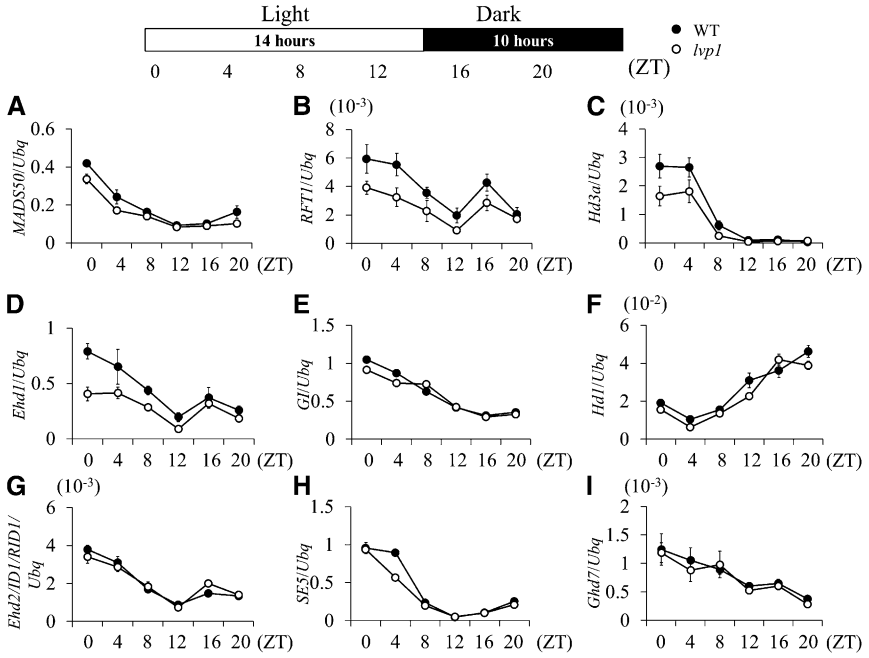

Figure 4. Diurnal Expression Differences of Flowering Time Genes between Wild-type and *lvp1* Plants Grown under LD Conditions.

Leaf samples were collected every 4 h from 30-d-old plants grown under LD conditions. Each point represents at least three biological replicates. *y* axis, relative transcript levels of flowering time genes normalized to those of rice *Ubiquitin*. Error bars indicate SD (n = 3 or more). WT, wild-type; ZT, Zeitgeber time. Primers are listed in Supplemental Table 1 online.

type plants. The heading date for wild-type plants grown in Beijing under natural LD conditions is ~70 d. Thus, to ensure that both wild-type and *lvp1* mutant plants used in the assay were at the vegetative stage, crude histone extracts were isolated from the top three leaves of 50-d-old plants. As loading control, the total amount of histone H3 was determined using a specific antibody. The intensities of immunoblot signals were measured by ImageJ software, and the relative abundance of each histone modification was determined (see Supplemental Figure 4 online). Compared with the wild type, the levels of H3K4me1, H3K4me2, H3K4me3, and H3K9me2 showed no obvious differences in the *lvp1-1* and *lvp1-2* mutants; however, the levels of H3K36me1, H3K36me2, and H3K36me3 were clearly reduced in both mutants (Figure 3C; see Supplemental Figure 4 online). It is interesting to note that the decrease in H3K36me2 levels in *lvp1* plants was more pronounced than that of the other two H3K36 methylation types (Figure 3C; see Supplemental Figure 4 online). In summary, lesions in *SDG724* lead to a global decrease of H3K36me1, H3K36me3, and especially H3K36me2 levels, indicating that SDG724 is a histone methyltransferase responsible for H3K36 methylation in rice.

SDG724 Is Required in the *MADS50-Ehd1-RFT1* Flowering Pathway under LD Conditions

Because the loss-of-function mutant *lvp1* had an extremely late-flowering phenotype under LD conditions, we determined whether the diurnal expression of major flowering time genes controlling the floral transition under LD photoperiods was affected. Leaf samples were collected every 4 h from 30-d-old plants grown under LD conditions, and expression of the genes was analyzed. Quantitative RT-PCR results showed that expression levels of *GI*, *Hd1*, and *SE5* changed little in *lvp1* plants; however, expression levels of *MADS50* and *Ehd1* were decreased in the *lvp1* mutant (Figure 4). Due to the decreased level of *Ehd1*, expression levels of *RFT1* and *Hd3a* were also lower in *lvp1* plants. This suggested that SDG724 activity probably affects the *Ehd1* but not the *Hd1* pathway of flowering. By contrast, *Ehd2*/*ID1*/*RID1* and *Ghd7* levels appeared to be unaffected by the *lvp1* mutation, suggesting that these genes are upstream of or parallel to *SDG724* in the gene regulatory network of flowering under LD conditions (Figure 4).

To provide further evidence that *MADS50*, *Ehd1*, *Hd3a*, and *RFT1* were downstream targets of *SDG724*, we examined the expression levels of those genes at four developmental stages (30, 60, 90, and 120 d after germination [DAG]) in plants grown in Beijing under natural LD conditions. Leaf samples were collected 2 h after dawn, when transcription of the putative downstream genes was at a high level. The results showed that compared with the wild type, transcript levels of the downstream genes were lower in the *lvp1* mutant and that transcript levels of the flowering time genes peaked at 60 DAG in wild-type plants (see Supplemental Figure 5 online). These observations further supported the conclusion that under LD conditions, SDG724 activity promoted *MADS50* transcription, which leads to upregulation of its downstream targets *Ehd1* and *FT-like* genes. Also notably, *MADS50* expression was only marginally changed in the controlled growth chamber under artificial LD conditions at

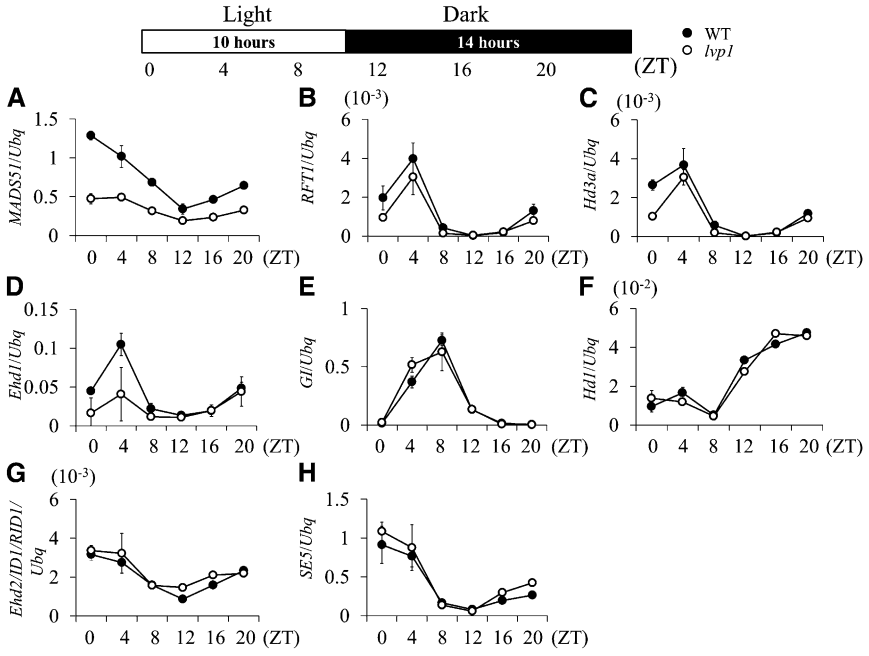

Figure 5. Diurnal Expression Differences of Flowering Time Genes between Wild-Type and *lvp1* plants Grown under SD Conditions.

Leaf samples were collected every 4 h from 25-d-old plants grown under SD conditions. Each point represents at least three biological replicates. *y* axis, relative transcript levels of flowering time genes normalized to those of rice *Ubiquitin*. Error bars indicate SD (*n* = 3 or more). WT, wild-type; ZT, Zeitgeber time. Primers are listed in Supplemental Table 1 online.

30 d (Figure 4) but considerably changed under natural LD conditions at 60 DAG (see Supplemental Figure 5 online), when the *lvp1* mutant and wild-type plants were all still vegetative.

SDG724 Promotes the *MADS51-Ehd1-Hd3a* Flowering Pathway under SD Conditions

Because *lvp1* mutants also flowered ~12 d later under SD conditions (see Supplemental Figure 1 online), we examined the diurnal expression levels of genes in the SD flowering pathway. Leaf samples were collected every 4 h from 25-d-old plants grown under SD conditions. *Ehd2*/*ID1*/*RID1*, *SE5*, *MADS51*, *GI*, *Ehd1*, *Hd1*, and *Hd3a* were analyzed as likely members of the SD flowering pathway (Hayama et al., 2003; Kim et al., 2007). Compared with wild-type plants, transcript levels of *MADS51* and *Ehd1* were decreased, correlating with lower expression levels of *Hd3a* in *lvp1* plants; however, there were no obvious alterations in *GI* and *Hd1* expression levels (Figure 5). These results suggested that *SDG724* promoted the flowering transition through *MADS51-Ehd1*, but not *GI-Hd1*. Additionally, no significant differences were observed in expression levels of *SE5* and *Ehd2*/*ID1*/*RID1* between the wild-type and *lvp1* mutant plants, indicating that these loci are not controlled by *SDG724*. Taken together, genes involved in the *MADS51-Ehd1* pathway may require *SDG724* for high expression, which affects expression levels of *Hd3a* under SD conditions.

Mutations in *SDG724* Delay Flowering Irrespective of Photoperiod

Lesions in *SE5*, which encodes a putative heme oxygenase in phytochrome chromophore biosynthesis of rice, produce an extremely early flowering phenotype and a complete lack of photoperiodic response (Izawa et al., 2000, 2002). The early flowering phenotype of *se5* mutants under both SD and LD conditions is mainly due to increased expression levels of *Ehd1* (Andrés et al., 2009). To investigate the role of *SDG724* in a photoperiod-insensitive background, *lvp1 se5* double mutants were created using a *se5* nonsense mutation in Nipponbare, the same genetic background as for the *lvp1* mutant (see Supplemental Figure 6 online). Under both SD and LD conditions, double mutant *lvp1 se5* plants flowered earlier than wild-type plants. However, in comparison to *se5*, the flowering time of *lvp1 se5* was delayed by ~6 d under both SD and LD conditions, suggesting that efficient upregulation of flowering time genes in the *se5* mutant background was partially dependent

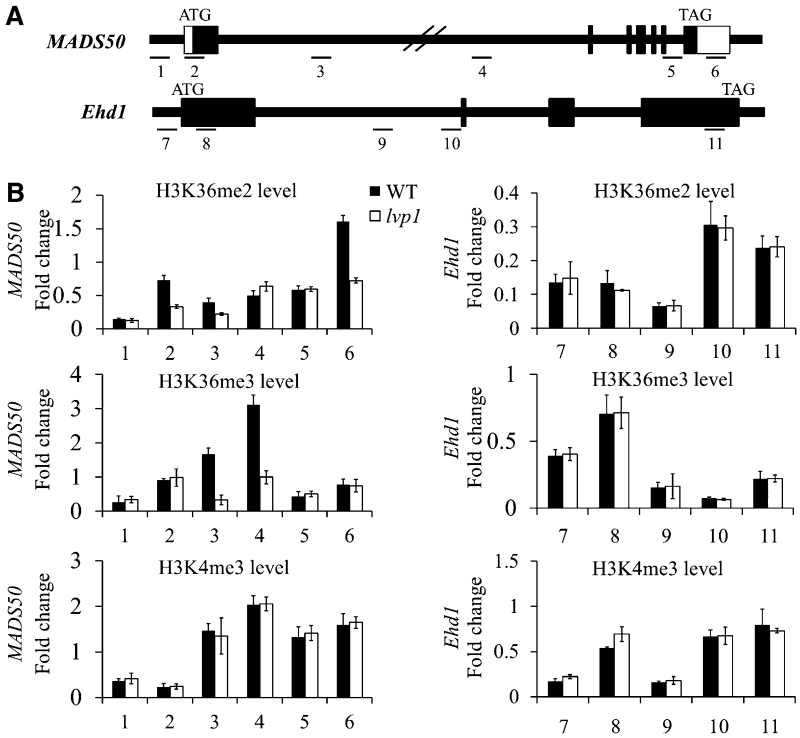

Figure 6. H3K36me2/3 Levels Are Reduced at the *MADS50* Locus in *lvp1* Mutant Plants.

ChIP samples were analyzed by quantitative PCR of six regions of *MADS50* and five regions of *Ehd1*. The rice *Actin1* gene was used as an internal control. The relative levels of specific histone H3 Lys methylation marks were calculated as the ratio of specific methylation marks over total H3 and normalized as the ratio over total H3 at *Actin1*; error bars indicate SD (n = 3 or more). Primers are listed in Supplemental Table 1 online.

(A) Genomic structure of the *MADS50* and *Ehd1* loci. Regions assayed in **(B)** are indicated by lines and numbered.

(B) ChIP analysis of H3K36me2/3 and H3K4me3 deposited on the chromatin of *MADS50* and *Ehd1* loci in wild-type (WT) and *lvp1* mutant plants.

on SDG724 activity. To test this, flowering time genes were analyzed, indicating that compared with the *se5* single mutant, expression levels of *MADS51*, *MADS50*, *Ehd1*, *Hd3a*, and *RFT1* were lower in *lvp1 se5* double mutant plants under both SD and LD conditions (see Supplemental Figures 7 and 8 online), suggesting that *SDG724* was necessary for full activation of *Ehd1* expression in the *lvp1 se5* double mutant. We speculate that in the *lvp1 se5* double mutant, *Ehd1* transcript levels are decreased due to reduced expression of *MADS50* and *MADS51* under both LD and SD conditions, thus causing a late flowering phenotype. By contrast, no obvious differences in *Hd1* expression levels between *se5* single and *lvp1 se5* double mutant plants were observed (see Supplemental Figures 7 and 8 online), further confirming that *SDG724* was independent of the *Hd1* associated pathway. Taken together, we show that lesions in *SDG724* delay flowering via the *Ehd1* flowering pathway, even in a genetic background lacking any degree of photoperiodic sensitivity.

Levels of H3K36me2/3 Are Decreased at the *MADS50* and *RFT1* Loci

The H3K36 methylation mark is generally associated with transcriptionally active chromatin states (Cazzonelli et al., 2009a; Liu et al., 2010). According to our results, the SDG724 protein functions as an H3K36 methyltransferase and promotes the expression of certain flowering time regulatory genes. This evidence prompted us to determine whether SDG724 was required for depositing H3K36 methylation marks onto the chromatin of the flowering time genes. We collected leaf samples of 50-d-old plants grown under Beijing natural LD conditions and samples of 25-d-old plants grown under artificial SD conditions in a growth chamber. At these two time points, both wild-type and *lvp1* plants were at the vegetative growth stage, and the transcripts of SDG724 target genes were all at lower levels in the *lvp1* mutant (Figure 4; see Supplemental Figure 5 online). Chromatin immunoprecipitation (ChIP) experiments were done using

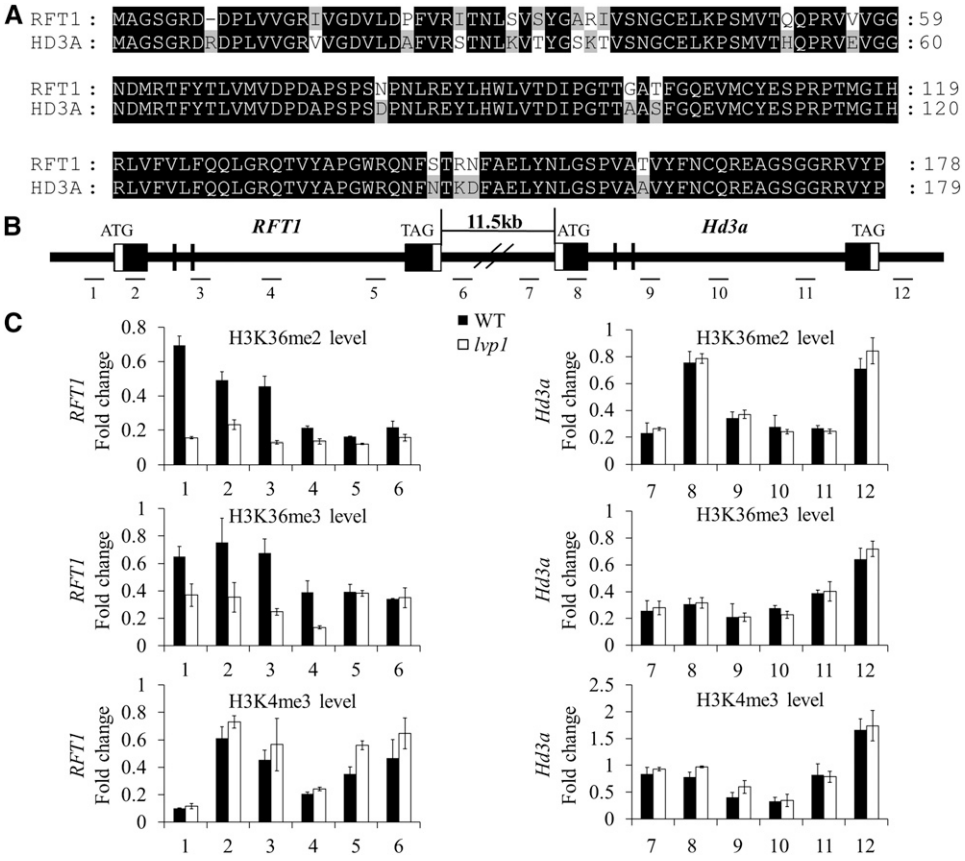

Figure 7. H3K36me2/3 Levels Are Severely Reduced at the *RFT1* Locus in *lvp1* Mutant Plants.

ChIP samples were analyzed by quantitative PCR of six different regions of each gene. The rice *Actin1* gene was used as an internal control. The relative levels of specific histone H3 Lys methylation marks were calculated as the ratio of specific methylation marks over total H3 and normalized as the ratio over total H3 at *Actin1*; error bars indicate SD (n = 3 or more). Primers are listed in Supplemental Table 1 online.

(A) Alignment of RFT1 and Hd3a protein sequences using ClustalX and GenDoc. Shades of black indicate conserved amino acids.

(B) Genomic structure of the *RFT1* and *Hd3a* loci on chromosome 6. Black boxes indicate exons, and white boxes indicate untranslated regions. Regions assayed in **(C)** are indicated by lines and numbered.

(C) ChIP analysis quantifying H3K36me2/3 and H3K4me3 levels in the chromatin of the *RFT1* and *Hd3a* loci in wild-type (WT) and *lvp1* mutant plants.

specific antibodies against H3K36me2 and H3K36me3 because their levels were globally decreased in *lvp1* mutants compared with the wild-type plants. As specificity and loading controls, antibodies against H3K4me3 and H3 were used, respectively. Two MADS box genes, *MADS50* and *MADS51*, were shown to be downregulated in the *lvp1* mutant. For the ChIP assay, a reduced H3K36me2/3 level was observed within the gene body region of *MADS50* in the *lvp1* mutant, which is in accordance with its decreased expression level (Figure 6). However, despite the low level of *MADS51* transcripts in the *lvp1* mutant under SD conditions, no obvious changes in H3K36me2/3 levels were detected between mutants and the wild type at the chromatin regions we examined (see Supplemental Figure 9 online). These results suggested different mechanisms account for the downregulation of the two MADS box genes *MADS50* and *MADS51* in the *lvp1* mutant, and only the expression of *MADS50* closely correlated with the chromatin modification levels of H3K36me2/3.

RFT1 and *Hd3a* encode two rice florigens and are closely linked in the genome, separated by only 11.5 kb. However, *RFT1* and *Hd3a* have functionally diverged to control the LD and SD flowering time pathways, respectively (Komiya et al., 2008). Our ChIP results showed that H3K36me2/3 levels of *RFT1* at most chromosomal regions examined were reduced in the *lvp1* mutant. However, only subtle changes in H3K36 methylation were detected on the chromatin of *Hd3a* under either SD or LD conditions (Figure 7; see Supplemental Figure 9 online). Thus, despite of their close chromosomal locations and high degree of similarity, SDG724 specifically affects H3K36me2/3 levels at the *RFT1*, but not the *Hd3a* locus. In addition, H3K36me2/3 levels at the *Ehd1* locus were also found to be unchanged in *lvp1* plants, even though its expression was downregulated in *lvp1* compared with wild-type plants (Figure 6). At all the loci examined, H3K4me3 levels were not significantly different between *lvp1* mutant and wild-type plants, which demonstrated a specific relationship between SDG724 and H3K36 methylation.

DISCUSSION

In this study, we identified *LVP1/SDG724*, as a rice histone methyltransferase gene. SDG724 is a class II SET domain protein and is constitutively expressed in various kinds of tissues (see Supplemental Figure 10 online). Members of this class, including SDG4, SDG8, and SDG25 in *Arabidopsis* and SDG725 in rice, were shown to have H3K36 methylation activities (Zhao et al., 2005; Cartagena et al., 2008; Dong et al., 2008; Xu et al., 2008; Cazzonelli et al., 2009b; Grini et al., 2009; Berr et al., 2010a; Ko et al., 2010; Sui et al., 2012). Our study demonstrated that SDG724 is a major factor for H3K36me3 deposition in rice for the following reasons. First, recombinant SDG724 protein can specifically methylate histone H3 in oligonucleosomes (Figures 3A and 3B), indicating that it has in vitro H3 HMTase activity; second, global levels of H3K36me1, H3K36me2, and H3K36me3 were all reduced in *lvp1* plants due to a lesion in *SDG724* (Figure 3C); third, lesions in *SDG724* specifically decreased the abundance of H3K36me2/3 marks at the *MADS50* and *RFT1* loci in *lvp1* plants (Figures 6 and 7).

In animals and yeast, H3K36 methylation recruits other histone modifiers, such as histone acetyltransferases or deacetylases, thus reflecting the complexity of transcriptionaln mechanisms (Liu et al., 2010). In addition, H3K36me3 also provides a splicing-related mark, which is involved in the histone modification status in *Caenorhabditis elegans* (Liu et al., 2010). In *Arabidopsis*, H3K36 methylation may participate in fine-tuning transcription levels at individual gene loci (Guo et al., 2010), but it is not clear whether the regulatory mechanisms of H3K36 methylation are shared by different species (Liu et al., 2010). The identification of the HMTase SDG724 as a major factor in H3K36 methylation in rice will facilitate future studies on how H3K36 methylation may affect chromatin structure and gene transcription in different organisms.

Although the flowering time genes *MADS50*, *MADS51*, *Ehd1*, *Hd3a*, and *RFT1* all displayed significant changes in transcriptional activity due to a lesion in *SDG724*, H3K36me2/3 levels were only decreased at *MADS50* and *RFT1* in *lvp1* mutant plants. *RFT1* and *Hd3a* are paralogs and members of the phosphatidylethanolamine binding protein gene family (Chardon and Damerval, 2005), and they appear to be the only two major florigen genes in rice (Tamaki et al., 2007; Komiya et al., 2008, 2009). However, several lines of evidence indicate that the functions of the two paralogs have diverged after duplication (Hagiwara et al., 2009). First, it was shown that *RFT1* but not *Hd3a* is the major florigen in the fields of northern Asia under natural LD growth conditions (Komiya et al., 2008). Second, the nucleotide sequences of *RFT1* are more highly differentiated in various rice accessions and have thus diverged more rapidly than *Hd3a* during rice evolution (Hagiwara et al., 2009). Third, it has been observed that the critical photoperiod for *RFT1* activation was not as fixed as that for *Hd3a* (Itoh et al., 2010). Taken together, this evidence indicates that *RFT1* and *Hd3a* are regulated by different genetic mechanisms, and our study provides new evidence for this interpretation. Because lesions in *SDG724* specifically lowered H3K36me2/3 levels in chromatin of the *RFT1* but not the *Hd3a* locus (Figure 7), we conclude that different biochemical mechanisms regulate the transcriptional

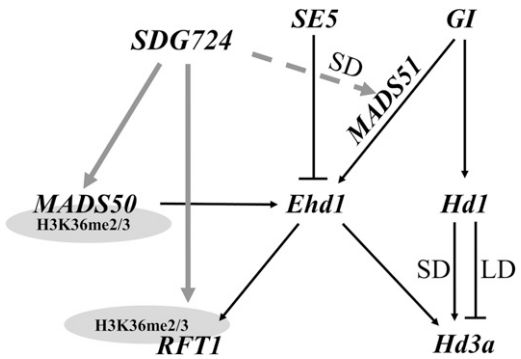

Figure 8. Proposed Model for the *SDG724* Flowering Pathway in Rice.

The black lines show previously identified pathways, and the gray lines indicate pathways added to this model from our study. *SDG724* is required for high expression of *MADS50*, *MADS51*, *Ehd1*, *RFT1*, and *Hd3a*, but influences H3K36me2/3 levels only in *MADS50* and *RFT1* chromatin.

activities of those two florigen genes. Similarly, our ChIP data also suggest that the two MADS box flowering time genes *MADS50* and *MADS51* may also have a similar epigenetic regulatory mechanism as *RFT1* and *Hd3a*. It will be interesting in future studies to examine whether other histone methylation states are needed to distinguish the roles of the two florigen genes in promoting the flowering transition in rice.

In rice, *Hd1* and *Ehd1* are two important integrators of the floral transition, and numerous genes controlling their expression have been identified (Komiya et al., 2009; Tsuji et al., 2011). Our results suggest that *SDG724* can affect expression levels of *Ehd1* via *MADS50*/*MADS51* and independent of *Hd1* (Figure 8). Furthermore, the double mutant *lvp1 se5* had lower expression levels of *Ehd1* than *se5* single mutant plants, confirming that *SDG724* is required for the *Ehd1* associated pathway (see Supplemental Figures 7 and 8 online). However, the *japonica* rice cultivar Taichung 65 carries loss-of-function alleles of both *Hd1* and *Ehd1*, but it flowers within the range of other accessions and serves as a commercial rice variety (Doi et al., 2004), suggesting that besides the *Hd1* and *Ehd1* pathways, other not yet identified regulatory mechanisms control the floral transition in rice (Doi et al., 2004; Hagiwara et al., 2009; Saito et al., 2009). Here, we propose that *SDG724* may affect the floral transition by activating *RFT1*, independently of the *Hd1* and *Ehd1* flowering promotion pathways (Figure 8). *SDG724* is responsible for global H3K36me2/3 methylation, which primarily correlates with transcriptionally active chromatin in rice, and lesions in *SDG724* reduced *RFT1* transcript levels in association with a reduction in the level of H3K36me2/3 marks at the *RFT1* locus. Thus, *SDG724* might directly control the H3K36me2/3 states of *RFT1* chromatin, and the *SDG724-RFT1* pathway may be part of a novel *Hd1* and *Ehd1* independent program for activating the floral transition in rice.

In rice, the floral transition is an important agronomic trait for climatic and regional adaptability, and studies on heading date are not only necessary for understanding the genetic mechanisms of flowering, but are also significant for agriculture. Although the northern limit for growing wild rice species is around 28°N, cultivated rice can be grown as far north as around 45°N (Izawa, 2007a). Thus, it is important to identify the floral transition mechanisms in rice under LD conditions, and our work provides evidence for a regulatory mechanism controlled by *SDG724* under LD conditions, which could also be useful for commercial rice improvement. Along those lines, a recently launched breeding program for growing rice at low latitudes with almost constant SD conditions aims to develop rice varieties with long basic vegetative growth phases (BVPs). Long BVPs allow rice plants to achieve sufficient vegetative growth by delaying flowering, thus producing high-yielding plants that can be used in double or triple cropping (Saito et al., 2009; Yuan et al., 2009). *Ef1* (*Ehd1*) was the first locus identified in this BVP breeding program (Yuan et al., 2009), which is in agreement with our study showing that *SDG724* promotes flowering mainly through *Ehd1* under SD conditions. Moreover, because lesions in *SDG724* still repress flowering in *lvp1 se5* double mutants, which are blind to photoperiod, *lvp1* mutant plants could be a useful genetic material for rice breeding to achieve long BVPs in areas with low latitudes.

METHODS

Plant Materials and Growth Conditions

Flowering mutants were screened under natural LD conditions in a paddy field at the experimental station of the Institute of Genetics and Developmental Biology in Beijing, China, in the summer of 2004. An F2 population from a cross between *lvp1* and Minghui 63 (*Oryza sativa* ssp *indica*) was used to map *LVP1* under natural LD conditions in Beijing. Materials used here were also planted in growth chambers in which artificial LD conditions consisted of 14 h light at 30°C followed by 10 h darkness at 28°C, and SD conditions consisted of 10 h light at 30°C followed by 14 h darkness at 28°C.

Map-Based Cloning

We generated a total of 121 simple sequence repeat and STS markers showing polymorphisms between the *lvp1* mutant and Minghui 63 for linkage analysis. The novel STS markers were designed based on the difference of DNA sequences between Nipponbare (*O. sativa* ssp *japonica*) and 9311 (*O. sativa* ssp *indica*) using the Primer Premier 5.0 software (http://www.premierbiosoft.com; Tong et al., 2009). The key mapping markers are listed in Supplemental Table 1 online.

Vector Construction and Plant Transformation

For complementation of the *lvp1* mutant, a 1346-bp 3′-downstream fragment was obtained by PCR using primers SDG724-C and inserted into the pCAMBIA1300 vector after digestion with *Bam*HI-*Pst*I. An 8567-bp genomic fragment containing the entire *SDG724* transcript was then removed from BAC clone AP006235 by digestion with *Bam*HI-*Sma*I and introduced into the pCAMBIA1300 vector carrying the 1346-bp 3′-downstream regions. The *lvp1* mutant was used as the recipient for transformation by an *Agrobacterium tumefaciens*–mediated method, as described previously (Liu et al., 2007).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted and quantitative RT-PCR was performed using the Bio-Rad CFX96 real-time system following a protocol as described previously (Yang et al., 2009). First-strand cDNA was synthesized from 2 μg total RNA. The rice *Ubiquitin* gene was used as an internal control in quantitative RT-PCR analysis, and all primers for the flowering genes analyzed are listed in Supplemental Table 1 online.

Protein Purification and HMTase Assays

The full-length cDNA of *SDG724* was amplified by PCR using primers SDG724-His-MBP (see Supplemental Table 1 online) and cloned into the pMCSG7 vector containing His-MBP tags. The *SDG724* fusion gene was expressed in *Escherichia coli* (*BL21* [*RIL*]; Invitrogen), and recombinant protein was purified using a His-Bind purification kit (EMD Chemicals). Recombinant protein was then cleaved using the tobacco etch virus protease, and the mature SDG724 protein without His-MBP tags was used for HMTase assays. HMTase assays were performed using tritium-labeled SAM (TRK865; Amersham Biosciences) to monitor the incorporation of tritium-labeled methyl groups into different histone substrates. After termination of the HMTase assay, the reaction mix was separated by SDS-PAGE gel electrophoresis, dried, and exposed on x-ray film (Y. Ding et al., 2007; Jacob et al., 2009).

Histone Extraction and Immunoblot Analysis

Crude histone extracts were made from 50-d-old leaves of wild-type and *lvp1* plants grown under natural LD conditions in Beijing, and immunoblot

analyses were performed as described (Guo et al., 2010). Detection of histone H3 protein was used as loading control. For each immunoblot analyzing a specific modification, the same membrane was stripped and blotted with an antibody against H3. Specific antibodies used were as follows: ab1791 (anti-H3; Abcam; 1:10,000 dilution), 07-436 (anti-H3K4me1; Upstate; 1:10,000 dilution), 07-030 (anti-H3K4me2; Upstate; 1:10,000 dilution), 07-473 (anti-H3K4me3; Upstate; 1:5000 dilution), 07-548 (anti-H3K36me1; Upstate; 1:3000 dilution), 07-369 (anti-H3K36me2; Upstate; 1:3000 dilution), and ab9050 (anti-H3K36me3; Abcam; 1:1000 dilution).

ChIP

Considering their different expression patterns, *MADS50*, *MADS51*, *Ehd1*, *Hd3a*, and *RFT1* were all at a high level in the *lvp1* mutant and wild type at Zeitgeber time 2, leaves of 50-d-old plants grown under Beijing natural LD conditions and 25-d-old plants grown under artificial SD conditions in a growth chamber were collected 2 h after dawn for ChIP experiments as previously described (Berr et al., 2010b). The antibodies 07-473 (anti-H3K4me3; Upstate), 07-369 (anti-H3K36me2; Upstate), ab9050 (anti-H3K36me3; Abcam), and ab1791 (anti-H3; Abcam) were used to pull down chromatin containing the specific methylation marks. Detection of *MADS50*, *MADS50*, *Ehd1*, *Hd3a*, and *RFT1* chromatin regions using quantitative PCR as described previously (Guo et al., 2010). The rice *Actin1* gene was used as an internal control for the quantitative PCR analysis, and all the primers used are listed in Supplemental Table 1 online.

Accession Numbers

Sequence data from this article can be found at http://www.chromdb.org/ under the following accession numbers: *Os09g13740* (*SDG724*, rice), *Os04g34980* (*SDG708*, rice), *Os02g34850* (*SDG725*, rice), *Os02g39800* (*SDG736*, rice), *At2g44150* (*SDG7*, *Arabidopsis*), *At3g59960* (*SDG24*, *Arabidopsis*), *At4g30860* (*SDG4*, *Arabidopsis*), *At1g77300* (*SDG8*, *Arabidopsis*), *At1g76710* (*SDG26*, *Arabidopsis*), *AF545814* (*SDG110*, maize), and *AY122273* (*SDG102*, maize). Other accession numbers, which can be found in National Center for Biotechnology Information databases, are listed in Supplemental Table 1 online.

Supplemental Data

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

Supplemental Figure 1. Heading Date Investigation of Wild-Type and *lvp1* Plants under SD and LD Conditions.

Supplemental Figure 2. Rescue of *lvp1* Phenotype by Transformation with *SDG724*.

Supplemental Figure 3. Phylogenetic Analysis of SDG724 Homologs in Plants.

Supplemental Figure 4. Quantification of Immunoblot Signals.

Supplemental Figure 5. Expression Levels of *MADS50*, *Ehd1*, *Hd3a*, and *RFT1* at Different Developmental Stages of LD Grown Plants.

Supplemental Figure 6. Analysis of Double Mutant *lvp1 se5* Plants.

Supplemental Figure 7. Expression Analysis of Flowering Genes in *se5* Single and *lvp1 se5* Double Mutant Plants Grown under SD Conditions.

Supplemental Figure 8. Expression Analysis of Flowering Genes in *se5* Single and *lvp1 se5* Double Mutant Plants Grown under LD Conditions.

Supplemental Figure 9. H3K36me2/3 Levels Are Not Affected at the *MADS51* and *Hd3a* Loci in *lvp1* Mutant Plants Grown under SD Conditions.

Supplemental Figure 10. Expression Pattern Analysis of *SDG724* in Different Tissues by Quantitative RT-PCR.

Supplemental Table 1. Primers Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Corresponding to the Phylogenetic Analysis in Supplemental Figure 3.

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AUTHOR CONTRIBUTIONS

C.S., J.F., and C.C. designed the experiments, analyzed the data, and wrote the article. J.F., J.T., and Q.Q. identified the mutant materials. C.S., J.F., and X.D. constructed and identified the double mutant *lvp1 se5*. C.S., J.F., and F.C. performed map-based cloning, vector construction, and plant transformation. C.S., F.Z., and L.L. performed RNA extraction and quantitative RT-PCR. C.S., T.Z., and G.Z. performed protein purification and HMTase assays, histone extraction, and immunoblot analysis. C.S., T.Z., B.X., and X.C. performed ChIP.

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