Ehd2, a Rice Ortholog of the Maize *INDETERMINATE1* Gene, Promotes Flowering by Up-Regulating *Ehd1*[1][C][W]

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Recent research into the flowering of rice (*Oryza sativa*) has revealed both unique and conserved genetic pathways in the photoperiodic control of flowering compared with those in Arabidopsis (*Arabidopsis thaliana*). We discovered an *early heading date2* (*ehd2*) mutant that shows extremely late flowering under both short- and long-day conditions in line with a background deficient in *Heading date1* (*Hd1*), a rice *CONSTANS* ortholog that belongs to the conserved pathway. This phenotype in the *ehd2* mutants suggests that *Ehd2* is pivotal for the floral transition in rice. Map-based cloning revealed that *Ehd2* encodes a putative transcription factor with zinc finger motifs orthologous to the *INDETERMINATE1* (*ID1*) gene, which promotes flowering in maize (*Zea mays*). *Ehd2* mRNA in rice tissues accumulated most abundantly in developing leaves, but was present at very low levels around the shoot apex and in roots, patterns that are similar to those of *ID1*. To assign the position of *Ehd2* within the flowering pathway of rice, we compared transcript levels of previously isolated flowering-time genes, such as *Ehd1*, a member of the unique pathway, *Hd3a*, and *Rice FT-like1* (*RFT1*; rice florigens), between the wild-type plants and the *ehd2* mutants. Severely reduced expression of these genes in *ehd2* under both short- and long-day conditions suggests that *Ehd2* acts as a flowering promoter mainly by up-regulating *Ehd1* and by up-regulating the downstream *Hd3a* and *RFT1* genes in the unique genetic network of photoperiodic flowering in rice.

Flowering is one of the fundamental events in the life cycle of many higher plants and is a very important trait for determining the ability of a species to adapt to various environmental conditions. Flowering time is largely determined by the timing of the transition from vegetative to reproductive growth and is controlled by both environmental signals and developmental programs. Photoperiod (i.e. daylength) is an important environmental signal that determines flowering time in plants and recent molecular genetic research in Arabidopsis (*Arabidopsis thaliana*) has revealed how plant sensitivity to photoperiod controls flowering time (Kobayashi and Weigel, 2008; Turck et al., 2008). It has been demonstrated that the *CONSTANS* (*CO*) transcription factor up-regulates the transcription of the *FLOWERING LOCUS T* (*FT*) gene in Arabidopsis leaves in response to induction by long daylength (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002; Valverde et al., 2004). FT protein then moves to the shoot apex, where it interacts with the transcription factor encoded by *FD* to activate genes that determine floral organ identity and consequently induces flowering (Abe et al., 2005; Wigge et al., 2005; Corbesier et al., 2007).

A genetic pathway similar to that in Arabidopsis is conserved in the photoperiodic control of flowering in rice (*Oryza sativa*), a short-day (SD) plant. *Heading date1* (*Hd1*) is one of the first flowering-related genes to have been cloned from a natural variant of rice (Yano et al., 2000). The Arabidopsis *CO* promotes flowering only under long-day (LD) conditions, whereas *Hd1* (a *CO* ortholog in rice) promotes flowering under SD conditions and represses it under LD conditions (Yano et al., 2000). Another flowering-related gene, *Hd3a*, is a rice ortholog of *FT* and is regulated by *Hd1* (Izawa et al., 2002; Kojima et al., 2002). Recently, Tamaki et al. (2007) demonstrated that the Hd3a protein functions as a mobile florigen-type flowering signal. Additionally, *Rice FT-like1* (*RFT1*), the closest homolog of *Hd3a*, may act redundantly to *Hd3a* as a floral promoter (Izawa et al., 2002; Kojima et al., 2002; Komiya et al., 2008). In Arabidopsis, the *CO*/*FT* module is regulated by *GIGANTEA* (*GI*), which is a component of the genes related to the circadian clock (Suarez-Lopez et al., 2001; Sawa et al., 2007). Similarly, it has been demonstrated that regulation of the *Hd1*/*Hd3a* module is mediated by *OsGI*, a rice ortholog of *GI* (Hayama et al., 2003). These findings reveal that a common floral induction pathway from *CO* to *FT* in photoperiodic control of flowering is conserved in both Arabidopsis and rice, but that the photoperiodic response has

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differentiated between these LD and SD (respectively) plants. On the other hand, *Early heading date1* (*Ehd1*) is a flowering time gene unique to rice that encodes a B-type response regulator, with no obvious counterpart in the Arabidopsis genome (Doi et al., 2004). *Ehd1* promotes floral transition preferentially under SD conditions, even in the absence of functional alleles of *Hd1*. Expression analysis revealed that *Ehd1* functions upstream of *Hd3a*, *RFT1*, and some MADS-box genes (Doi et al., 2004). More recently, *Ghd7*, which encodes a CCT (CO, CO-LIKE, and TIMING OF CAB1)-domain protein was isolated from natural variations in rice (Xue et al., 2008). *Ghd7* affects levels of *Ehd1* and *Hd3a* transcripts, but does not affect *Hd1* mRNA levels. *Ghd7* represses *Ehd1* and *Hd3a* expression under LD conditions, thereby delaying flowering. Thus, two independent floral pathways are present in rice: the conserved *Hd1* pathway and a unique *Ehd1* pathway that may integrate environmental photoperiod signals into the expression of *FT-like* genes (Izawa, 2007).

Although the recent rapid accumulation of knowledge about the genetic control of flowering in rice has been largely based on the analysis of natural variations (Yano et al., 2001; Kojima et al., 2002; Doi et al., 2004; Xue et al., 2008), a large part of the control pathway remains to be analyzed compared with Arabidopsis, in which analysis of this pathway has progressed mainly by using flowering mutants (for review, see Koornneef et al., 1998; Kobayashi and Weigel, 2008; Turck et al., 2008). Therefore, to comprehensively understand the genetic control of flowering in rice, we require more analysis of mutants as well as natural variants.

In this study, we discovered an *ehd2* mutant that flowers extremely late compared with wild-type plants under both SD and LD conditions. The presence of this phenotype in the *ehd2* mutants suggested that the wild-type gene (*Ehd2*) essentially acts as a flowering promoter. In this article, we describe molecular cloning of *Ehd2* and the gene's role in the control of photoperiodic flowering in rice. No significant morphological aberration was observed in the vegetative and reproductive organs of the *ehd2* mutants. Map-based cloning revealed that *Ehd2* encodes a putative transcription factor with zinc finger motifs, which is orthologous to the *INDETERMINATE1* (*ID1*) gene in maize (*Zea mays*). Mutations in *ID1* have severe effects on the floral transition (Singleton, 1946; Colasanti et al., 1998): The late-flowering phenotype demonstrated that *ID1* is essential for normal floral transition. The *ID1* gene is expressed specifically in developing leaves (Colasanti et al., 1998; Kozaki et al., 2004; Wong and Colasanti, 2007). The floral induction pathway mediated by *ID1* may be unique to monocots because no clear *ID1* ortholog is present in Arabidopsis (Colasanti et al., 2006). Rice, which is closely related to maize, has a putative *ID1* ortholog with a leaf-specific accumulation of protein similar to that in maize (Colasanti et al., 2006). However, its role in controlling flowering time remains to be clarified. Expression analysis of genes related to rice flowering in the *ehd2* mutant and the wild type, and genetic interactions between *Hd1* and *Ehd2*, demonstrated that *Ehd2* promotes floral transition mainly by up-regulating *Ehd1* and genes downstream of *Ehd1*, such as *Hd3a* and *RFT1*. These results indicate the critical role of a unique genetic flowering pathway in monocotyledonous plants such as rice.

RESULTS

Phenotypes of the *ehd2* Mutant

The *ehd2* mutant was identified as a late-flowering variant of the M_2 plants from a γ-ray-mutagenized line of rice cv Tohoku IL9 (subsp. *japonica*). Flowering time of the *ehd2* mutants (177.0 ± 13.2 d) was delayed by more than 77 d compared with the wild-type plants (99.4 ± 1.1 d) under natural-day (ND) conditions (Fig. 1A).

To test whether the flowering times of the *ehd2* mutants differed among photoperiodic conditions, we grew the mutants and corresponding wild-type plants under SD conditions (10 h light/14 h dark) and LD conditions (14.5 h light/9.5 h dark). Under SD conditions, flowering time of the *ehd2* mutants was 206.5 d, an increase of 145 d compared with the wild-type plants (61.5 ± 1.3 d; Fig. 1A). Under LD conditions, the *ehd2* mutants never flowered during more than 365 d, whereas the wild-type plants flowered at 73.7 ± 2.1 d (Fig. 1A). The difference in flowering time in the wild-type plants under the SD and LD conditions was small (12 d; Fig. 1A). Thus, the *ehd2* mutants showed extremely late flowering compared with the wild-type plants under both conditions, although the mutation had a more severe effect on flowering time under LD conditions.

To examine whether a reduction in growth rate or a prolonged plastochron might have caused the late flowering in the *ehd2* mutants, we next compared the leaf emergence rate between the *ehd2* mutants and the wild-type plants until 144 d. The wild-type plants flowered after 12 leaves had emerged under SD conditions and after 15 leaves had emerged under LD conditions (Fig. 1B). Before flowering of the wild-type plants, the leaf emergence rate of the *ehd2* mutants was almost indistinguishable from that of the wild-type plants under both SD and LD conditions (Fig. 1B). Under both conditions, the *ehd2* mutants had developed 20 leaves by 144 d after germination. By the time the wild-type plants flowered, the leaf size and plant height in the *ehd2* mutants were similar to those in the wild-type plants under ND conditions (Fig. 1C). The *ehd2* mutants eventually flowered under ND and SD conditions (Fig. 1A). No significant morphological aberration was evident in the *ehd2* mutants, although the inflorescences with ripened seeds were smaller than those of the wild-type plants (Fig. 1D). Thus, the growth rate and development of the inflorescences were not affected by the *ehd2* mutation. These results demonstrate that *Ehd2* controls the floral transition in rice, but not its growth rates.

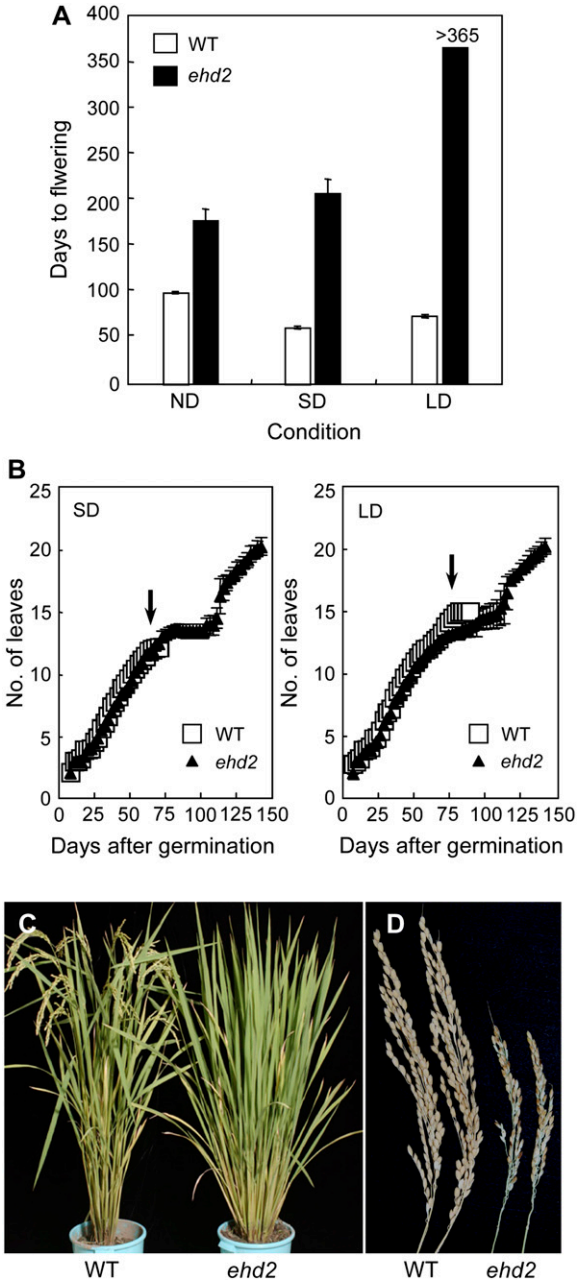

Figure 1. Flowering date phenotypes of the *ehd2* mutant compared with those of the wild-type plant (cv Tohoku IL9). A, Days to flowering of the wild-type plants and the *ehd2* mutants under different photoperiodic conditions (means ± SD, $n = 10$). B, Comparison of leaf emergence rates between wild-type plants and *ehd2* mutants under SD and LD conditions during development (means ± SD, $n = 5$). Leaf emergence rate was calculated according to the measurements described by Itoh et al. (1998). Average days to flowering of the wild-type plants are shown by an arrowhead. C, Wild-type plant (left) and *ehd2* mutant (right) under ND conditions after flowering of the wild-type plant. D, Inflorescences of a wild-type plant (left) and the *ehd2* mutant (right).

Ehd2 Encodes a Putative Transcription Factor

We then performed map-based cloning of *ehd2*. The *ehd2* mutant was first crossed with an *indica* cultivar (Guang Lu Ai 4), and the resultant F_1 was backcrossed with cv Guang Lu Ai 4. It was easy to obtain sufficient DNA marker polymorphisms between *japonica* and *indica* rice. The *ehd2* phenotype segregated as a monogenic recessive trait in the BC_1F_2 population (Supplemental Fig. S1). Because the mutant phenotype behaved as a complete recessive, the mutation appears to have been caused by an absence of *Ehd2* function. We next performed bulked segregant analysis using plants with normal and mutant phenotypes from the BC_1F_2 population. The result of the analysis revealed that a simple sequence repeat (SSR) marker (RM6124) on chromosome 10 was linked to the gene for the mutant phenotype (Fig. 2A). Furthermore, a high-resolution linkage analysis demonstrated that *ehd2* is delimited within a 13.9-kb genomic region between two single-nucleotide polymorphisms, SNP-1 and SNP-2, on chromosome 10 (Fig. 2B; Supplemental Table S1). An SSR marker (SSR-1) showed cosegregation with the mutant phenotype (Supplemental Table S1). In this candidate region, two putative proteins, a zinc finger protein (Os10g0419200) and a heat shock transcription factor (Os10g0419300), were annotated in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp; zinc finger and heat shock transcription factor, respectively; Fig. 2B). Comparison of the sequence of the candidate region between the *ehd2* mutant and the wild-type *Ehd2* plants revealed a 4-bp insertion within the second exon of the putative zinc finger protein in the *ehd2* mutant (Fig. 2C), resulting in a premature stop codon in the open reading frame (ORF). No other nucleotide polymorphisms were observed in this 13.9-kb candidate region. A homology search using tBLASTn software (http://blast.ddbj.nig.ac.jp/top-j.html) revealed that the putative zinc finger protein is a rice homolog of the maize ID1 protein (accession no. AF058757) that is involved in the transition to flowering (Colasanti et al., 1998). The two proteins shared 58% amino acid identity over the entire peptide sequence, but fasta software (http://fasta.ddbj.nig.ac.jp/top-j.html) indicated that the identity between the putative zinc finger domains reached 82%.

The 3,496-bp *Ehd2* gene consisted of three exons and two introns (Fig. 2C). The deduced sequence of 475 amino acids in the protein had a nuclear localization signal motif (KKKR) and four zinc finger motifs (two C2H2-type and two C2HC-type), previously designated as the ID domain (Fig. 2D; Supplemental Fig. S2; Kozaki et al., 2004). In addition, the deduced C-terminal peptide sequence of the Ehd2 protein was nearly identical to the 19 C-terminal amino acids of maize ID1 (17/19 amino acids). A phylogenetic analysis among the deduced amino acid sequences of rice, maize, and the Arabidopsis ID domain revealed that *Ehd2* is a rice ortholog of the maize *ID1* gene (Supplemental Fig. S3; also inferred by Colasanti et al., 2006). A synteny conservation between the harbored regions of *Ehd2* (rice chromosome 10) and *ID1* (maize chromosome 1) also supported the results of the phylogenetic analysis (Rice Chromosome 10 Sequencing Consortium, 2003).

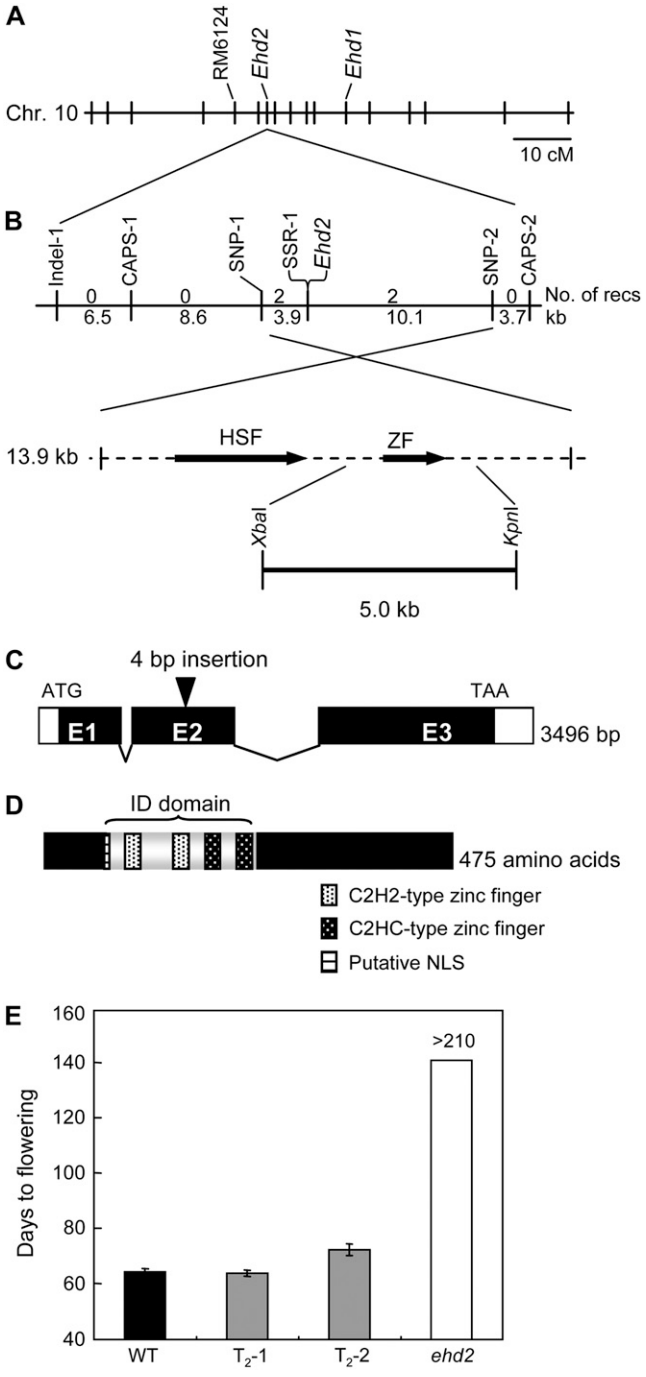

Figure 2. Results of the map-based cloning of *Ehd2*. A, Location of *Ehd2* on chromosome 10. B, High-resolution linkage map of *Ehd2* (n = 2,047). A 5.0-kb genomic fragment digested by *Xba*I and *Kpn*I from cv Nipponbare was introduced into the *ehd2* mutant. HSF, Heat shock factor; ZF, zinc finger protein. C, Structure of *Ehd2*. E1, E2, and E3 represent exons. White boxes represent the 5′-untranslated region (left) and the 3′-untranslated region (right), respectively. The structure of the mRNA was determined by RACE. D, Structure of the Ehd2 protein. E, Days to flowering of two independent T_2 lines homozygous for the *Ehd2* transgene from cv Nipponbare under SD (10 h light/14 h dark) conditions (means ± SD, n = 10).

To demonstrate that the *ehd2* mutant phenotype was caused by a loss-of-function mutation in the putative zinc finger protein, we complemented *ehd2* by transforming it with the corresponding cv Nipponbare genomic fragment; cv Nipponbare had a sequence for the putative *Ehd2* ORF identical to that of cv Tohoku IL9. The 5.0-kb fragment consisted of a 0.9-kb upstream sequence, the putative coding region, and a 0.7-kb downstream sequence digested by *Xba*I and *Kpn*I. In these transgenic plants, the *Ehd2* phenotype was restored under SD conditions (Fig. 2E). These results confirmed that *Ehd2* encodes the ID1 ortholog.

Ehd2 mRNA Is Abundant in the Developing Leaf

Previous studies in maize revealed that *ID1* mRNA appears exclusively in developing leaves (Colasanti et al., 1998; Wong and Colasanti, 2007). Therefore, we examined the level of *Ehd2* transcripts in several rice tissues (Fig. 3A) by means of quantitative real-time (RT)-PCR under LD conditions. A clear gradient in the level of *Ehd2* transcripts was observed among the rice tissues (Fig. 3B). *Ehd2* mRNA accumulated most abundantly in developing leaves within the leaf sheath (1.55×10^{-2}). In the blades of the developed leaves (DL), it gradually decreased as the leaves aged to 0.63×10^{-2} in DL1, 0.23×10^{-2} in DL2, and 0.20×10^{-2} in DL3. Levels were low around the shoot apex (0.06×10^{-2}) and transcripts were undetectable in the roots. These results demonstrate that rice *Ehd2* mRNA accumulated most abundantly in developing leaf tissues, as maize *ID1* has been observed to do, but was also

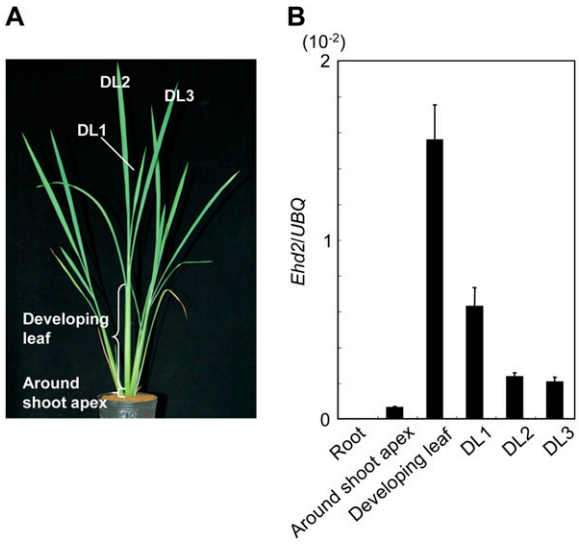

Figure 3. *Ehd2* mRNA expression in different tissues of rice. A, Wild-type plant 30 d after germination grown under LD conditions (14.5 h light/9.5 h dark). B, Quantitative RT-PCR results for *Ehd2* mRNA in different tissues. Values are shown as means ± SD of three independent experiments. DL, Developed leaf; UBQ, ubiquitin. Tissue samples were collected at 2 h after dawn. Data are representative of two independent biological replicates. [See online article for color version of this figure.]

present in mature leaves, although at much lower levels.

Ehd2 Promotes Flowering by Up-Regulating *Ehd1*, *Hd3a*, and *RFT1*

To identify potential downstream genes that are regulated by *Ehd2*, we examined the transcript levels of five flowering-related genes (*Ehd2*, *Hd1*, *Ehd1*, *Hd3a*, and *RFT1*) by means of quantitative RT-PCR. Leaf samples were collected from 3-week-old and 30-d-old plants grown under SD and LD conditions, respectively. The developmental stage of these plants was about 40 d before flowering.

We found that the *ehd2* mutant and the wild type both carried a defective *Hd1* allele (accession no. AB433218) derived from the *japonica* cv Sasanishiki. The defective allele had a 43-bp deletion and a 36-bp insertion in the first exon compared with the functional cv Nipponbare *Hd1* allele (accession no. AB041837), resulting in a premature stop codon (Fig. 4A). Nonetheless, the *Hd1* mRNA expression was observed in the wild-type plants and the mutants under both SD and LD conditions with a clear diurnal change (Supplemental Fig. S4). However, the level of the *Hd1* transcripts in the *ehd2* mutants was reduced under both SD and LD conditions compared with that of the wild-type plants. The results suggested that *Ehd2* up-regulates *Hd1* mRNA expression.

In the wild-type plants under SD conditions, the level of *Ehd2* transcripts started to increase after dusk and reached a peak before dawn (Fig. 4B). Then the *Ehd2* transcript level decreased once and increased again just before dawn. The *Ehd2* transcript level decreased gradually after dawn. Thus, *Ehd2* mRNA expression showed a clear diurnal change. The patterns of accumulation of *Ehd1*, *Hd3a*, and *RFT1* mRNAs appeared to parallel *Ehd2* expression, with a short delay, suggesting transcriptional regulation of these genes by *Ehd2*, although these transcripts remained abundant during the daytime, gradually decreasing until dusk. In contrast, *Ehd2* mRNA in the *ehd2* mutants remained at very low levels all day, with no sign of diurnal variation, possibly as a result of nonsense-mediated decay of the mRNA. Levels of *Ehd1*, *Hd3a*, and *RFT1* mRNAs decreased dramatically to nearly undetectable levels in the *ehd2* mutants. Under LD conditions, *Ehd2* mRNA showed diurnal changes in the wild-type plants and, to a much lesser extent, in the *ehd2* mutants (Fig. 4B). The pattern of accumulation of *Ehd1*, *Hd3a*, and *RFT1* in the wild-type plants was apparently more synchronized with *Ehd2* expression under LD conditions than under SD conditions, except that a second peak of *RFT1* expression was apparent during the daytime. In the *ehd2* mutants, the accumulation of *Ehd1*, *Hd3a*, and *RFT1* mRNAs was also repressed, at a significantly lower level, as was the case under SD conditions. These results demonstrated that *Ehd2* up-regulates the transcription of *Ehd1* and of the downstream *FT-like* genes, *Hd3a* and *RFT1*, under both SD and LD conditions, thereby promoting the floral transition.

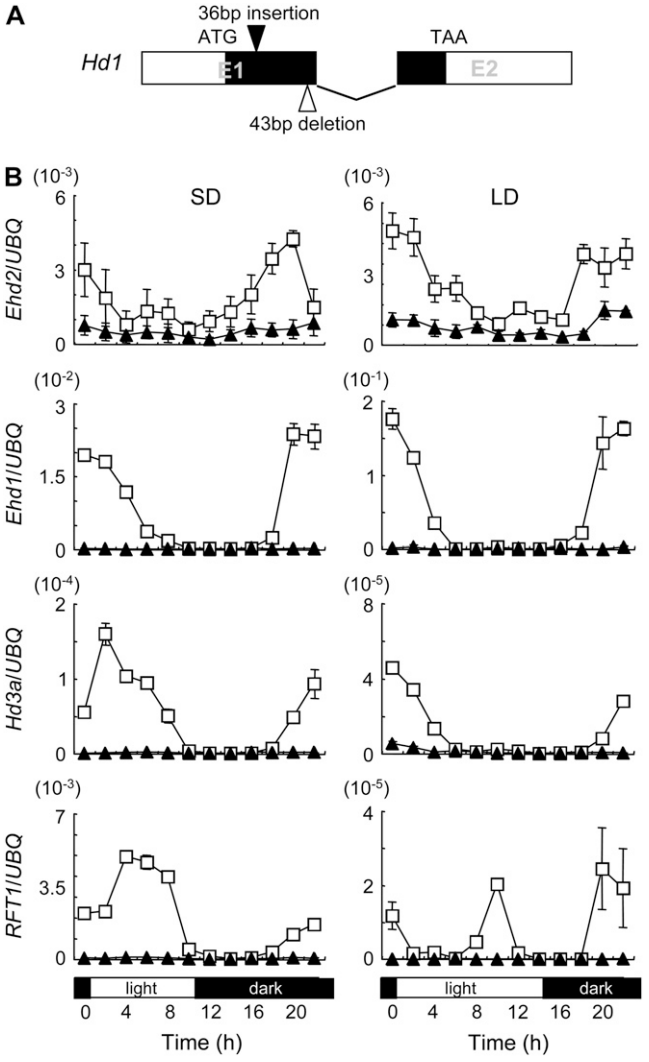

Figure 4. *Ehd2* up-regulates *Ehd1*, *Hd3a*, and *RFT1* (increases the level of their transcripts). A, Defective cv Tohoku IL9 allele of *Hd1*. E1 and E2 represent exons. White boxes represent the 5′- untranslated region (left) and the 3′- untranslated region (right), respectively. B, Diurnal changes in levels of *Ehd2*, *Ehd1*, *Hd3a*, and *RFT1* transcripts under SD (10 h light/14 h dark) and LD (14.5 h light/9.5 h dark) conditions. UBQ, Ubiquitin. White squares represent wild-type plants; black triangles represent the *ehd2* mutants. Developing leaves were harvested every 2 h. Values are shown as mean ± SD of three independent experiments. Data are representative of two independent biological replicates.

To further examine whether *Ehd2* regulates any other genes, we performed a microarray analysis of wild-type plants and *ehd2* mutants grown under LD conditions, with approximately 44,000 rice gene probes. Leaf samples were collected from 30-d-old plants 2 h after dawn. As expected, the level of *Ehd2* transcripts was dramatically less in the *ehd2* mutants than in the wild-type plants (Supplemental Table S2). The difference in levels of *Ehd1* transcripts between the

wild-type plants and the *ehd2* mutants showed the greatest down-regulation among the genes we examined (Supplemental Table S2). In addition to transcripts of *Ehd1*, the transcript levels of putative *NO APICAL MERISTEM* (*NAM*) protein domain-containing protein (Os08g0200600; Supplemental Fig. S5) and of *α-L-arabinofuranosidase/β-D-xylosidase isoenzyme* (*ARA-I*; Os04g0640700) were also greatly reduced in the *ehd2* mutants, but the roles of these proteins in floral induction is unknown, and further analysis is needed. The results of this analysis strongly support the hypothesis that *Ehd2* up-regulates *Ehd1* and that *Ehd1* is the primary downstream gene of the Ehd2 protein.

Levels of *Ehd2* Transcripts and Expression of Downstream Genes during Development

To examine the accumulation of *Ehd2*, *Ehd1*, *Hd3a*, and *RFT1* mRNAs during development, we harvested developed leaves from wild-type plants and *ehd2* mutants at different developmental stages and analyzed them by means of quantitative RT-PCR. The samples were collected 2 h after dawn under SD and LD conditions. In the wild-type plants under SD conditions, *Ehd2* mRNA was observed by 1 week after germination and reached a peak by 2 weeks (Fig. 5). Subsequently, the level gradually decreased, but transcripts were detected continuously at low levels even after flowering of the wild-type plants (about 9 weeks). The accumulation of *Ehd1* mRNA was detected at 2 weeks after germination, and increased greatly thereafter to reach a peak at 8 weeks. *Hd3a* mRNA was also observed at 2 weeks, then began to increase, and reached a peak at 6 weeks. *RFT1* mRNA was present at very low levels until 4 weeks, then began to increase, and reached a peak at 6 weeks. After flowering, levels of *Ehd1*, *Hd3a*, and *RFT1* mRNAs decreased. Under LD conditions in wild-type plants, the level of *Ehd2* transcripts was low during all developmental stages (Fig. 5), although transcription was detected from at least 1 week after germination. *Ehd1*, *Ehd3a*, and *RFT1* mRNAs were less abundant than under SD conditions, although transcription increased after 4 weeks.

In contrast, the transcription of *Ehd2* was very low in the *ehd2* mutants, and *Ehd1*, *Hd3a*, and *RFT1* transcripts were almost undetectable throughout all stages of development under both SD and LD conditions. These results further suggest that *Ehd2* functions upstream of *Ehd1* and of the *FT-like* genes.

Ehd2 Functions Upstream of *Hd1*

Because cv Tohoku IL9 (the wild-type of the *ehd2* mutant) carried a defective allele at the *Hd1* locus, the functional relationship between *Ehd2* and *Hd1* remained unconfirmed (Fig. 4A). Therefore, we screened *Tos17*-induced mutant lines (http://tos.nias.affrc.go.jp/~miyao/pub/tos17) and found an *ehd2* mutant with a *Tos17* insertion in cv Nipponbare, which carries a

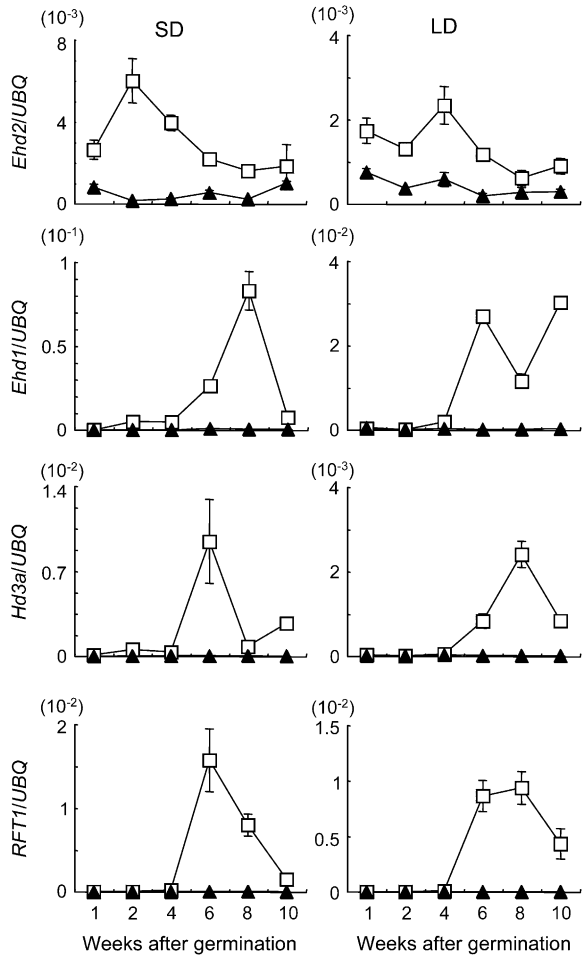

Figure 5. Change in levels of *Ehd2*, *Ehd1*, *Hd3a*, and *RFT1* transcripts during development under SD (10 h light/14 h dark) and LD (14.5 h light/9.5 h dark) conditions. UBQ, Ubiquitin. White squares represent wild-type plants; black triangles represent *ehd2* mutants. Developing leaves were harvested 2 h after dawn. Values are shown as mean ± SD of three independent experiments. Data are representative of two independent biological replicates.

functional allele at the *Hd1* locus (Yano et al. 2000). A sequence analysis of the flanking *Tos17* insertion revealed that the retrotransposon was inserted in the third exon of *Ehd2* (Fig. 6A).

Under ND conditions, the wild-type plants (cv Nipponbare) flowered at 116.6 ± 2.8 d and the *Tos17*-induced mutants of *Ehd2* (hereafter referred to as *ehd2-Tos17*) flowered at 144.1 ± 4.3 d without significant morphological aberration (Fig. 6B). We grew the *ehd2-Tos17* mutants and the wild-type plants under SD and LD conditions also. Under SD conditions, the wild-type plants flowered at 47.5 ± 1.1 d and the *ehd2-Tos17* mutants flowered at 132.7 ± 4.3 d. Under LD conditions, the wild-type plants flowered at 80.6 ± 3.4 d, but the *ehd2-Tos17* mutants did not flower for more than 365 d (Fig. 6B), as was the case for the *ehd2* mutants of cv Tohoku IL9 (Fig. 1A). The flowering time of the *ehd2-Tos17* mutants was significantly delayed (by more

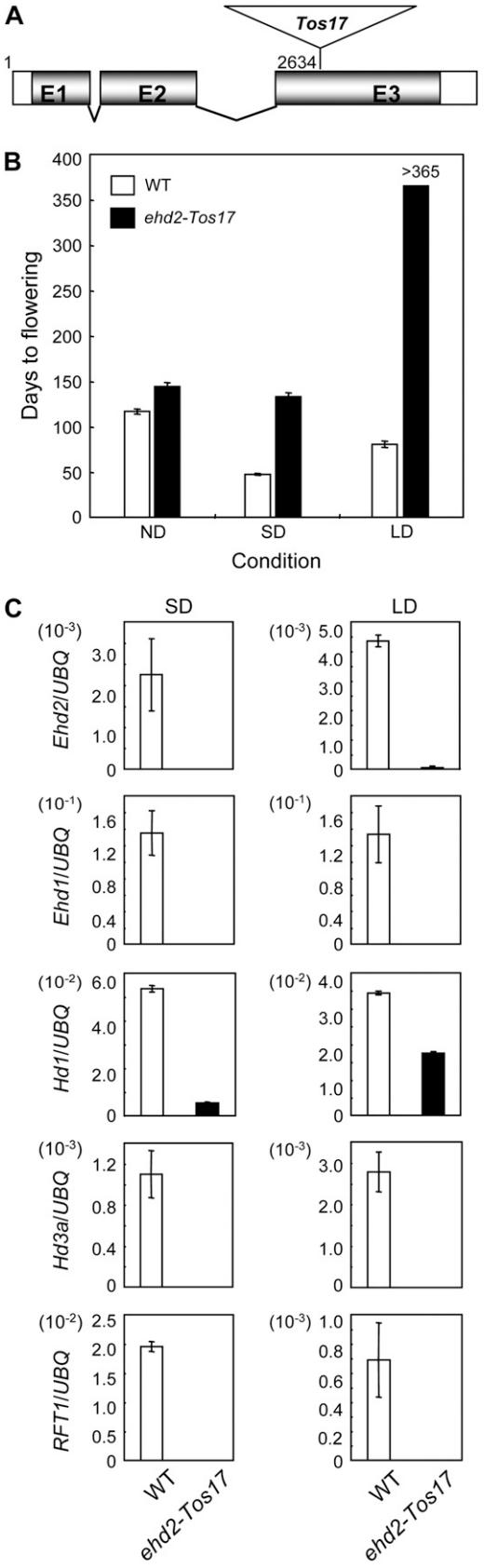

Figure 6. Characteristics of the *ehd2-Tos17* mutants. A, Diagram of the *ehd2* mutant disrupted by *Tos17* (i.e. *ehd2-Tos17*). B, Days to flowering of wild-type plants (cv Nipponbare) and of the *ehd2-Tos17* mutants under different daylength conditions (means ± SD, $n = 10$). C, Levels of *Ehd2*, *Ehd1*, *Hd1*, *Hd3a*, and *RFT1* transcripts in the *ehd2-Tos17* mutants under SD and LD conditions. Values are shown as means ± SD of three independent experiments. UBQ, Ubiquitin. Developing leaves were harvested 2 h after dawn under SD conditions for analysis of *Ehd2*, *Ehd1*, *Hd3a*, and *RFT1*, and after dusk for *Hd1*. Data are representative of two independent biological replicates.

than 85 d) compared with the wild-type plants under SD conditions. However, as in the case of the *ehd2* mutants of cv Tohoku IL9, the *ehd2-Tos17* mutants grown under SD conditions flowered dramatically earlier than those under LD conditions, suggesting that some unidentified gene other than *Hd1* and *Ehd1* can promote flowering under SD conditions in the *ehd2* mutants (Fig. 6B).

We further examined the levels of *Hd1* transcripts in the *ehd2-Tos17* mutants and the wild-type plants. First, we analyzed the accumulation of *Ehd2* and *Ehd1* mRNAs to confirm that the late-flowering phenotype was caused by the *Tos17*-induced mutation of *Ehd2* and the consequent reduced abundance of *Ehd1* mRNA. The leaf samples were harvested 2 h after dawn from plants that had been grown for 4 weeks under SD and LD conditions and expression analysis was performed. *Ehd2* and *Ehd1* transcripts were abundant in the wild-type plants, but were nearly undetectable in the *ehd2-Tos17* mutants (Fig. 6C). This result confirmed that the late flowering was caused by the *ehd2* mutation. Next, we compared *Hd1* mRNA expression between the *ehd2-Tos17* mutants and the wild-type plants. The leaf samples were harvested 6 h after dusk under SD conditions or at 1.5 h after dusk under LD conditions because it has been reported that the accumulation of *Hd1* mRNA increases after dusk (Izawa et al., 2002; Kojima et al., 2002). Contrary to our expectations, *Hd1* mRNA decreased in the *ehd2-Tos17* mutants under both photoperiodic conditions, although the degree of reduction was less dramatic than that for *Ehd1* mRNA (Fig. 6C). In addition, no accumulation of *Hd3a* or *RFT1* mRNAs was detected in the *ehd2-Tos17* mutants under either photoperiodic condition (Fig. 6C). These results demonstrate that *Ehd2* functions upstream of *Ehd1*. Because the reduction of *Hd1* mRNA accumulation in the *ehd2-Tos17* mutants was not severe compared with the reduction of *Ehd1* mRNA (Fig. 6C), *Hd1* appears to be functional in the *ehd2-Tos17* mutants. However, we could not see clear differences in *Ehd2* action between the cv Tohoku IL9 and cv Nipponbare backgrounds.

DISCUSSION

In this article, we cloned a rice ortholog of the maize *ID1* gene, *Ehd2*, and demonstrated that it plays a critical role in the photoperiodic control of flowering in rice. Comparison of mRNA accumulation between the

ehd2 mutants and wild-type plants (cv Tohoku IL9, carrying a deficient allele of *Hd1*) revealed that *Ehd2* function is required for the expression of *Ehd1* and of downstream *FT-like* genes under both SD and LD conditions (Figs. 4B and 5). Furthermore, expression analysis to compare *Tos17*-induced mutants of *Ehd2* with wild-type plants (cv Nipponbare carrying a functional allele of *Hd1*) revealed that *Ehd2* could up-regulate *Hd1* (Fig. 6C). However, *Hd1* may remain functional in the *ehd2-Tos17* mutant because the reduction of *Hd1* mRNA accumulation in the mutants was less severe than the reduction of *Ehd1* mRNA (Fig. 6C). In this situation, flowering was severely delayed in the *ehd2-Tos17* mutant under SD conditions (Fig. 6B). Therefore, *Hd1* may not compensate for the late flowering caused by the *ehd2-Tos17* mutation. Instead, *Ehd2* and *Hd1* may act additively because the *ehd2-Tos17* mutants with a cv Nipponbare background flowered sooner than the *ehd2* mutants with a cv Tohoku IL9 background. We cannot yet confirm this because we did not compare quantitative trait loci (QTL) between the two backgrounds. Note that either *Hd1* or *Ehd1* alone can promote rice flowering under SD conditions (Doi et al., 2004). On the basis of these results, we conclude that *Ehd2* plays a pivotal role in the promotion of floral transition in rice under both inductive SD conditions and noninductive LD conditions (Fig. 7). It is noteworthy that an as-yet unidentified photoperiodic genetic pathway may operate in rice under SD conditions because both the *ehd2* mutant and the *ehd2-Tos17* mutant flowered under these conditions, but not under LD conditions, suggesting that the mutant plants became more sensitive to photoperiod than the wild-type plants.

A diurnal change in *Ehd2* mRNA accumulation was observed under both SD and LD conditions, although we did not define whether this pattern was controlled by a circadian clock or by an acute response to light signals (Fig. 4B). In addition, *Ehd2* mRNA did not exhibit any photoperiodic responses because the expression of *Ehd1* did not appear to be affected much by photoperiod under our study conditions.

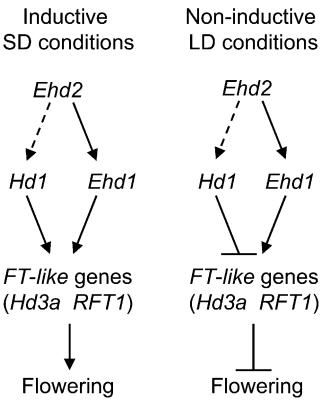

Figure 7. Schematic representation of the role of *Ehd2* in the genetic control of flowering in rice. Pathways suggested by the results of this study are shown by dashed lines.

During development, the accumulation of both *Ehd1* and *Hd3a* mRNAs was observed at 2 weeks under SD conditions, although it was low level (Fig. 5). The mRNA accumulation of these genes might follow the rise of *Ehd2* mRNA level and might be related to floral transition because, under SD conditions, the floral transition of wild-type plants (cv Tohoku IL9) should occur at about 3 weeks after germination. On the other hand, the level of *Ehd1* and *Hd3a* mRNAs clearly reached a peak after the floral transition, although we do not have any knowledge about the increase in level of *Ehd1* and *Hd3a* mRNAs after floral transition for now. Under LD conditions, the mRNA accumulation pattern of *Ehd2* and the downstream genes showed a similar trend as under SD conditions with short delay.

Ehd2 is not the only gene that functions upstream of *Ehd1*. Recently, it has been reported that a type I MADS box gene, *OsMADS51*, also up-regulates *Ehd1* and subsequently activates *Hd3a* mRNA expression (Kim et al., 2007). In *osmads51* mutants, flowering time was delayed by about 2 weeks compared with wild-type plants under inductive SD conditions, but little change was observed under noninductive LD conditions (Kim et al., 2007). Therefore, it is likely that *OsMADS51* preferentially promotes flowering under SD conditions (Kim et al., 2007). The microarray analysis in our study suggested that *OsMADS51* is not downstream of *Ehd2* because we observed no significant change in *OsMADS51* expression between the *ehd2* mutants and the wild-type plants. On the other hand, if *OsMADS51* was upstream of *Ehd2*, the level of *Hd1* transcripts would be altered in loss-of-function mutants of *OsMADS51* because *Ehd2* functions upstream of *Hd1* (Fig. 6C). However, there was no apparent change in the abundance of *Hd1* mRNA between the *osmads51* mutants and the wild-type plants (Kim et al., 2007). Therefore, it is very likely that *OsMADS51* follows a distinct pathway from that of *Ehd2* and up-regulates *Ehd1* mRNA expression. More recently, it was reported that *Ghd7* is likely to down-regulate *Ehd1* under LD conditions via a different pathway from the *Ehd2* pathway (Xue et al., 2008). These findings demonstrate that *Ehd1* expression is regulated by multiple pathways in the photoperiodic control of flowering.

In maize, two genes other than *ID1* that play a role in flowering have been cloned and characterized thus far: *delayed flowering1* (*dlf1*; Muszynski et al., 2006) and *Vegetative to generative transition1* (*Vgt1*; Salvi et al., 2007). The *dlf1* mutation has less effect on flowering than *id1* mutations (Muszynski et al., 2006). *DLF1* encodes a bZIP transcription factor that is homologous to Arabidopsis FD, suggesting that DLF1 interacts with a maize FT-like partner to induce flowering (Muszynski et al., 2006). However, the FT-like partner has not yet been shown in maize. *Vgt1* had previously been known as a QTL for flowering time and was shown to be confined to an approximately 2-kb non-

coding region by map-based cloning (Salvi et al., 2007). It functions as a cis-acting regulatory element positioned about 70 kb upstream of an *APETALA2*-like transcription factor (termed *ZmRap2.7*). The *Vgt1* region is likely to have been evolutionarily conserved across maize, sorghum (*Sorghum bicolor*), and rice (Salvi et al., 2007).

The orthologous relationship between rice *Ehd2* and maize *ID1*, which show a very high identity (82%) between their zinc finger domains, had been previously inferred by Colasanti et al. (2006) and supported by evidence of synteny conservation (Rice Chromosome 10 Sequencing Consortium, 2003). However, the function of the rice ortholog had not yet been demonstrated at the time of our study. Our study demonstrated that rice *Ehd2* also acts as a flowering promoter. In addition, tissue-specific expression of orthologous genes in young leaves was likely to be similar (Fig. 3B; Colasanti et al., 1998, 2006; Wong and Colasanti, 2007). These results reveal that the functions of rice *Ehd2* and maize *ID1* are roughly conserved with respect to their role in flowering promotion and tissue-specific expression. On the other hand, it is likely that some functional differences between them are present. First, it has been reported that severe *id1* mutants perturb the normal transition to floral development and produce aberrant inflorescences with vegetative characteristics (Galinat and Naylor, 1951; Colasanti et al., 1998). Such a morphological aberration was not observed in the *ehd2* mutants in this study (Fig. 1, C and D). Certainly, the *ehd2* mutants produced smaller inflorescences than those of the wild-type plants (Fig. 1D), but the production of small inflorescences is generally observed in rice plants that are grown under noninductive LD conditions and that consequently have a long vegetative duration. Therefore, we hypothesize that rice *Ehd2* essentially acts as a flowering promoter. Maize *ID1* may also be involved in floral development, but with a different form of genetic control from that in rice. The plant architectures of the two species differ in that rice develops bisexual flowers, whereas maize develops spatially separated unisexual male and female flowers (Bommert et al., 2005). Such a morphological difference should be caused by developmentally distinct patterns of genetic control. Therefore, species specificity of genetic control of floral development may result in the phenotype differences produced by mutations of rice *Ehd2* and maize *ID1*. Identification of the genes involved in floral development downstream of both *Ehd2* and *ID1* is needed to clarify this issue.

The amount of *ID1* mRNA and its protein showed no obvious diurnal changes even if the maize plants were subjected to different light and dark cycles (Wong and Colasanti, 2007). Microarray analysis revealed that none of the *CO-like* and *FT-like* genes showed significant differential expression between the wild-type plants and the *id1* mutants, suggesting that *ID1* expression is not under photoperiodic control (Coneva et al., 2007). Instead, Wong and Colasanti (2007) proposed that *ID1* expression is developmentally regulated because *ID1* mRNA was abundant in immature leaves, but less abundant in greening leaf tips and mature albino leaves. From those findings, Coneva et al. (2007) suggested that *ID1* functions in an autonomous floral induction pathway that is distinct from the photoperiod-based induction pathway, although they did not completely rule out the possibility of involvement of the ID1 protein in the *CO/FT* regulatory pathway. Because the expression pattern is not altered by daylength, rice *Ehd2* and maize *ID1* appear to behave similarly. However, rice *Ehd2* is certainly integrated in the photoperiod induction pathway (Figs. 4B, 5, and 6C), whereas maize *ID1* may not be. We could not define any maize B-type response regulator (RR) as an *Ehd1* ortholog from any database-registered protein. Maize ZmRR9 (AB062095) shares high identity between the receiver and *Golden2*, Arabidopsis *RESPONSE REGULATOR*, and *Chlamydomonas* regulatory protein of P-starvation acclimatization response DNA-binding domains of ZmRR9 and those of Ehd1 (43% and 65%, respectively), but other parts of ZmRR9 were not conserved in Ehd1 (Asakura et al., 2003; Ito and Kurata, 2006). Such functional differentiation in the downstream genes between rice *Ehd2* and maize *ID1* may affect their photoperiodic responses. Both rice and maize, which originated from low-latitude regions, are basically SD plants (Thomas and Vince-Prue, 1997). However, modern rice cultivars exhibit photoperiod sensitivity, whereas maize cultivars are considered to be essentially day neutral (Coneva et al., 2007). This question is intriguing because of its relevance in the process of divergence in the floral induction pathways of rice and maize.

In summary, *Ehd2* promotes floral transition by up-regulating *Ehd1* primarily and by up-regulating the downstream *Hd3a* and *RFT1* genes (Fig. 7), demonstrating that *Ehd2* is a key factor in the genetic network that controls photoperiodic flowering in rice. Like *Ehd1*, there is no obvious ortholog of rice *Ehd2* or maize *ID1* in Arabidopsis. Moreover, some functional differences are likely to be present even between the orthologous genes of rice and maize in their floral induction pathways. Further clarifying how these differences contribute to the control flowering time in rice, maize, and Arabidopsis will improve our understanding of the diversification of photoperiodic control of flowering in higher plants at a molecular level.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Two *japonica* rice (*Oryza sativa*) cv Tohoku IL9 and cv Nipponbare, were used as the wild-type controls; cv Tohoku IL9 is a near-isogenic derivative of the *japonica* cv Sasanishiki. The *ehd2* mutant was identified in an M_2 generation of γ-irradiated cv Tohoku IL. The *indica* cv Guang Lu Ai 4 was used for mapping of the *ehd2* locus. A *Tos17*-induced mutant of *Ehd2* was obtained from the National Institute of Agrobiological Sciences of Japan (*Tos17* mutant panel project; http://tos.nias.affrc.go.jp/~miyao/pub/tos17). Plants were grown in a controlled-growth cabinet (Especmic TGEH-9) under SD conditions (10 h light/14 h dark; 28°C for 12 h and 24°C for 12 h) or LD conditions (14.5 h light/

9.5 h dark; 28°C for 12 h and 24°C for 12 h) at 60% relative humidity. Light was provided by metal halide lamps (300- to 1,000-nm spectrum, 500 μmol m^{-2} s^{-1}). For evaluation under ND conditions, plants were also grown from mid-April in a paddy field in Tsukuba, Japan. The daylengths during vegetative growth were 13.1 h at germination (mid-April), 14.1 h at 30 d after germination (mid-May), 14.6 h at 60 d (mid-June), 14.4 h at 90 d (mid-July), 13.5 h at 120 d (mid-August), and 12.4 h at 150 d (mid-September).

Map-Based Cloning

To map the *ehd2* locus, the *ehd2* mutant was first crossed with the *indica* cv Guang Lu Ai 4 to obtain sufficient DNA marker polymorphisms and the resultant F_1 population was backcrossed with cv Guang Lu Ai 4. We then performed bulked segregant analysis by pooling equal amounts of DNA from 10 BC_1F_2 plants with a late-flowering phenotype (homozygous for the recessive allele at *ehd2*) or 10 BC_1F_2 plants with a normal flowering phenotype (heterozygous or homozygous for the dominant *Ehd2* allele). A total of 93 SSR markers, which were distributed evenly across all 12 chromosomes, were selected to examine SSR marker polymorphism between the late-flowering phenotype with the *ehd2* mutant and the normal flowering phenotype in cv Guang Lu Ai 4. For the high-resolution mapping, the progeny (2,047 plants) of heterozygotes of the initial mapping population were used. To test the complementation of *Ehd2*, we cloned a 5.0-kb genomic fragment of cv Nipponbare, which was digested by *Xba*I and *Kpn*I and transformed into the pPZP2H-lac binary vector (Fuse et al., 2001). The resultant plasmid was then introduced into the *ehd2* mutant by means of *Agrobacterium*-mediated transformation (Hiei et al., 1994). Homozygous T_2 progeny derived from single-copy T_0 transformants were grown under SD conditions and their flowering time was recorded. The structure of the mRNA of *Ehd2* was determined by means of RACE using the Marathon cDNA amplification kit (CLONTECH).

Identification of the *Tos17*-Induced Mutant of *Ehd2*

To identify the *Tos17*-induced mutation of *Ehd2*, we extracted genomic DNA using a cetyltrimethylammonium bromide method (Murray and Thompson, 1980) from the selfed progeny of *Ehd2* heterozygotes and used PCR analysis to examine the cosegregation of *Tos17* with the late-flowering phenotype by genotyping with a *Tos17*-specific primer (5′-AGGAGGTTGCTTAGCAGTGAAACG-3′) in combination with two *Ehd2*-specific primers (5′-TTGTTCATGCCTGCAGGAAG-3′ and 5′-AATTTGCATTATGGCTTGATCTTC-3′). We identified plants that carried a homozygous insertion for *Tos17* and cosegregated with the late-flowering phenotype (Supplemental Fig. S6). The sequence flanking the *Tos17* insertion was determined by direct sequencing of the PCR products.

Quantitative RT-PCR Analysis of Gene Expression

Total RNA was extracted from leaves by using the SDS-phenol method (Shirzadegan et al., 1991). Total RNA (2.5 μg) was primed with the dT_{18} primer by using the first-strand cDNA synthesis kit (Amersham Biosciences) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed as previously described (Kojima et al. 2002). cDNA corresponding to 50 ng of total RNA was used as the template for each TaqMan PCR reaction (Applied Biosystems). At least three PCR reactions using the same templates were performed to obtain average values for the expression levels. The PCR conditions were 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. To quantify *Ehd2* mRNA expression, we used the specific primers 5′-CGACAATAGCTCGATCGCC-3′ and 5′-AAGCCCGAAGCTGACACTGT-3′ and the probe 5′-CGATGTCATGGTGGCTGCAGGTG-3′. *Hd1*, *Ehd1*, *Hd3a*, *RFT1*, and *UBQ* mRNAs were quantified by using previously described primers and probes (Kojima et al., 2002 for *Hd1*, *Hd3a*, and *UBQ*; Doi et al. 2004 for *Ehd1* and *RFT1*). For copy number standards, quantified fragments of cloned cDNA were used.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB359195, AB359196, AB359197, and AB359198.

Supplemental Data

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

Supplemental Figure S1. Distribution of days to flowering under ND conditions in BC_1F_2 population (n = 109) from the cross between the *ehd2* mutant and cv Guang Lu Ai 4.

Supplemental Figure S2. Alignment of deduced amino acid sequences of rice Ehd2 and maize ID1.

Supplemental Figure S3. Phylogenetic comparison of ID domain amino acid sequences from rice, maize, and Arabidopsis.

Supplemental Figure S4. Diurnal changes in levels of *Hd1* transcripts under SD (10 h light/14 h dark) and LD (14.5 h light/9.5 h dark) conditions.

Supplemental Figure S5. Expression analyses of Os08g0200600 (NAM protein domain containing protein).

Supplemental Figure S6. Distribution of days to flowering under ND conditions in the selfed progeny (n = 48) of the *Tos17*-induced mutants heterozygous for *Ehd2*.

Supplemental Table S1. Genetic markers used in the delimitation of candidate genomic region of *Ehd2*.

Supplemental Table S2. Summary of microarray data.

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