Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1*

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Two evolutionarily distant plant species, rice (*Oryza sativa* L.), a short-day (SD) plant, and *Arabidopsis thaliana*, a long-day plant, share a conserved genetic network controlling photoperiodic flowering. The orthologous floral regulators—rice *Heading date 1* (*Hd1*) and *Arabidopsis CONSTANS* (*CO*)—integrate circadian clock and external light signals into mRNA expression of the *FLOWERING LOCUS T* (*FT*) group floral inducer. Here, we report that the rice *Early heading date 1* (*Ehd1*) gene, which confers SD promotion of flowering in the absence of a functional allele of *Hd1*, encodes a B-type response regulator that might not have an ortholog in the *Arabidopsis* genome. *Ehd1* mRNA was induced by 1-wk SD treatment, and *Ehd1* may promote flowering by inducing *FT-like* gene expression only under SD conditions. Microarray analysis further revealed a few MADS box genes downstream of *Ehd1*. Our results indicate that a novel two-component signaling cascade is integrated into the conserved pathway in the photoperiodic control of flowering in rice.

[*Keywords*: Photoperiodism; flowering time; two-component signaling]

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The gene network underlying photoperiodic flowering consists of photoreceptors, circadian clock systems, and floral integrator genes (Izawa et al. 2002; Mouradov et al. 2002; Simpson and Dean 2002; Yanovsky and Kay 2002; Hayama et al. 2003). Molecular genetic studies have revealed that orthologous genes in rice (*Oryza sativa*), a short-day (SD) plant, and *Arabidopsis thaliana*, a long-day (LD) plant, such as *Heading date 1* (*Hd1*)/*CONSTANS* (*CO*) and *Hd3a*/*FLOWERING LOCUS T* (*FT*), are implicated in the regulation of photoperiodic flowering (Kardailsky et al. 1999; Kobayashi et al. 1999; Samach et al. 2000; Yano et al. 2000; Izawa et al. 2002; Kojima et al. 2002).

The molecular basis of day-length measurement in both species was recently explained according to a physiology-based model, termed the external coincidence model, which was first proposed in the 1930s (Izawa et al. 2002; Yanovsky and Kay 2002; Hayama and Coupland 2003). First, daily light/dark cycles set the phase of circadian clocks. Then, the circadian clocks express *Hd1*/*CO* mainly during the subjective night. In rice, *Hd1* inhibits floral transition by suppressing *FT-like* genes, including *FTL*, *Hd3a*, and *RFT1*, on long days, possibly interacting with Pfr phytochromes produced during the daytime. On short days, the longer duration of darkness may lead to a gradual decrease in levels of Pfr phytochrome during the night, resulting in alteration of the function of *Hd1* from a suppressor to an activator of *FT-like* genes at dawn (Izawa et al. 2002). Therefore, *Hd1* promotes flowering only under SD conditions. In *Arabidopsis*, long days cause *CO* expression at dusk, although short days do not. Recently, it was revealed that the day-length-dependent *CO* expression was caused through interaction mediated by *FKF1* (*FLAVIN-BINDING*, *KELCH REPEAT*, *F-BOX 1*) between blue-light signal transduction and circadian clocks (Imaizumi et al. 2003). *CRYPTOCHROME 2* (or *PHYTOCHROME A*)-mediated light signaling makes the CO protein an activator of *FT* at dusk (Yanovsky and Kay 2002). Therefore, *CO* promotes flowering under LD conditions. Thus, the regula-

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tion of *FT* group genes by *Hd1/CO* plays a central role in photoperiodism of both SD and LD plants.

Regardless of the important role of *Hd1*, this model does not fully explain photoperiodic flowering in rice, because *Hd1*-deficient strains exhibit a significant delay of flowering under continuous-light conditions compared with SD conditions, and still preferentially express *FT-like* genes at daytime under SD conditions (Izawa et al. 2002). It has also been shown that *Hd2*, another flowering-time quantitative trait locus (QTL) of rice, contributes to flowering responses to photoperiod independently of *Hd1* (Yano et al. 2001).

To better understand the molecular mechanisms of photoperiodic flowering in rice, we studied a *japonica* cultivar, Taichung 65 (T65), which exhibits a broad regional adaptability because of its reduced response to photoperiod (Nishida et al. 2001). In the present study, we show that T65 contains loss-of-function alleles of *Hd1* and *Ehd1* (*Early heading date 1*, formerly *Ef*(t); Doi and Yoshimura 1998), both of which play important roles in photoperiodic flowering of rice. We demonstrate that *Ehd1* can function as a floral inducer independently of *Hd1* and encodes a B-type response regulator. In addition, we analyzed four alleles of *Ehd1* to determine a functional nucleotide polymorphism that is related to flowering phenotypes, because we earlier identified the *Ehd1* locus as a QTL. We also reveal that *Ehd1* induces mRNA expression of *FT-like* genes after SD treatment, indicating that a novel two-component signaling cascade is integrated in the conserved photoperiodic flowering pathway in rice. Microarray analysis and RT–PCR assays revealed several genes downstream of *Ehd1*, suggesting that *Ehd1* signaling is specific to the control of photoperiodic flowering.

Results

Ehd1 *can promote flowering independently of* Hd1

The *Ehd1* gene was originally identified as a flowering-time QTL on rice chromosome 10 by using a cross-combination between T65 and an accession of African rice (*Oryza glaberrima* Steud. accession no. IRGC104038; Doi et al. 1998). The *O. glaberrima* allele of *Ehd1* (*Ehd1-gla*) is dominant and confers early flowering (Doi and Yoshimura 1998). This gene was detected as a QTL by using a cross-combination between T65 and Nipponbare. Single-point QTL analysis revealed that Nipponbare allele of *Ehd1* (*Ehd1-Nip*) confers early flowering (Fig. 1A). In addition, no major QTL near the *Ehd1* locus was found in a Nipponbare × Kasalath (an *indica* cultivar) cross; the only nearby QTL was a minor one, *Hd14* (Yano et al. 2001). Therefore, T65 may carry a loss-of-function allele of *Ehd1* (*ehd1-T65*), whereas *O. glaberrima*, Nipponbare, and Kasalath may contain functional *Ehd1* alleles (*Ehd1-gla*, *Ehd1-Nip*, and *Ehd1-Kas*).

To further characterize the function of *Ehd1* in photoperiodic flowering of rice, we generated the nearly isogenic line NIL(*Ehd1-gla*), in which the *Ehd1-gla* allele was introgressed into T65 by six rounds of backcrossing. Comparison of flowering time between T65 and NIL(*Ehd1-gla*) demonstrated that *Ehd1* confers early flowering, especially under SD condition (10 h light/14 h darkness, or 10L:14D; Fig. 1B,C).

We detected another QTL at the *Hd1* locus in the T65 × Nipponbare cross (Fig. 1A), in which the Nipponbare allele of *Hd1* was functional (Yano et al. 2000). This led us to analyze the genomic DNA sequence of *Hd1* in T65. We found that a retroelement-like fragment was inserted into the second exon, and the T65 allele of *Hd1* seemed to be a loss-of-function allele (Fig. 1D). Supporting this, *Hd1* mRNA was severely reduced in T65, probably by nonsense-mediated decay (Fig. 1E). To further confirm the deficiency of *Hd1* in T65, we transformed the functional Nipponbare allele of *Hd1* (*Hd1-Nip*) into T65 to generate the T65 + *Hd1* transgenic line. *Hd1-Nip* in T65 clearly promoted flowering under SD condition (10L:14D) and delayed flowering dramatically under LD condition (14.5L:9.5D; Fig. 1F). This is consistent with previous results (Yano et al. 2000; Izawa et al. 2002) in which *Hd1* functioned as a floral inducer under SD and as a floral repressor under LD. We confirmed that NIL(*Ehd1-gla*) contains the defective T65 allele of *Hd1* (Fig. 1G). Taken together, this genetic evidence clearly indicates that *Ehd1* can function to promote flowering in rice, independently of *Hd1*.

Ehd1 *encodes a B-type response regulator*

To confirm the *Hd1*-independent photoperiodic flowering pathway in rice, we performed map-based cloning of *Ehd1* (Fig. 2A–D). High-resolution mapping revealed that *Ehd1* is in a 16-kb genomic region on the BAC clone OSJNBa0071K18 (Fig. 2B). Transformation of T65 with the corresponding Kasalath genomic fragments verified that *Ehd1* encodes a B-type response regulator (RR; Hwang et al. 2002) of a 341 amino acid protein (Fig. 3A,B). It has a receiver domain at its N terminus and a GARP (*Golden2*, *Arabidopsis RESPONSE REGULATOR* [*ARR*], and *Chlamydomonas* regulatory protein of P-starvation acclimatization response [*Psr1*]) DNA-binding motif (Riechmann et al. 2000) in the middle portion (Fig. 3B–D).

To identify functional nucleotide variations corresponding to flowering phenotypes, we determined the genomic DNA sequences of *Ehd1* from T65, Kasalath, and *O. glaberrima* and obtained that of Nipponbare from GenBank (accession no. AC027038). Comparison of the deduced amino acid sequences of these four *Ehd1* alleles revealed that one amino acid was changed to Arg in the conserved residue of the GARP domain in the defective T65 allele, whereas the other three functional alleles had Gly at the corresponding position (Fig. 3D). The other amino acid changes are not restricted to T65 (Supplementary Fig. 1). We next used gel mobility-shift assays to compare the in vitro DNA-binding ability of the GARP domains of Ehd1-gla and ehd1-T65. Because the GARP domain of Ehd1 shares significant homology with that of ARR1, the same probes for ARR1 were used for this binding assay (Sakai et al. 2000). The results demonstrated

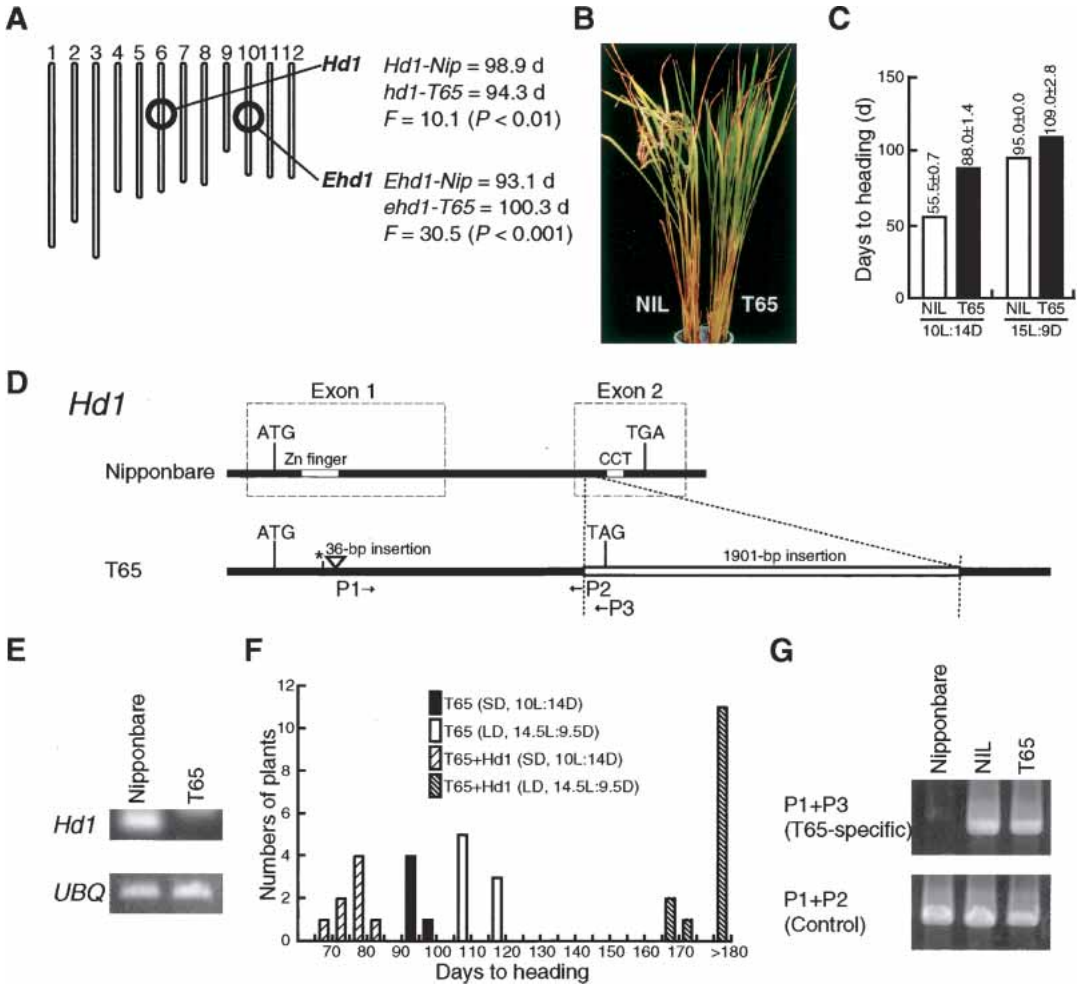

Figure 1. Genetic analysis of *Ehd1* and *Hd1*. (*A*) Major quantitative trait loci (QTLs) identified in 89 recombinant inbred lines derived from a cross between T65 and Nipponbare. Columns are chromosomes. Average of days to heading of each genotype and *F* values of single point QTL analyses at the *Hd1* and *Ehd1* loci are shown to the *right* of the rice linkage map. (*B*) Photograph of 80-day-old plants of NIL(*Ehd1-gla*) (*left*) and T65 (*right*) grown under SD (9L:15D) condition. (*C*) Days to heading of NIL(*Ehd1-gla*) and T65 under SD (10L:14D) and LD (15L:9D) conditions. (*D*) Allelic variation in *Hd1*. The genomic sequences of the functional Nipponbare and defective T65 alleles are shown schematically. The open triangle and asterisk indicate an insertion and a nucleotide substitution, respectively. T65 has a 1901-bp insertion in exon 2 that results in a premature stop codon (TAG) ahead of the CCT motif. P1, P2, and P3 are primers used in *E* and *G*. (*E*) mRNA levels of *Hd1* in Nipponbare and T65. RT–PCR was performed with the combination of primers P1 and P2. *UBQ* (ubiquitin) is shown as a control. (*F*) Days to heading of T65 and a T_2 transgenic line of T65 carrying the functional *Hd1-Nip* allele (T65 + *Hd1*). Similar results were obtained using independent transgenic lines. (*G*) NIL(*Ehd1-gla*) carries the defective *hd1* allele from T65. T65-allele-specific bands were amplified with primers P1 and P3 from genomic DNA. Controls amplified with P1 and P2 are also shown.

that the DNA-binding ability of the *ehd1-T65* allele was greatly reduced (Fig. 3E). Therefore, this Gly-to-Arg mutation may be crucial for the late-flowering phenotype of T65.

It is noteworthy that we could not define any *Arabidopsis* B-type RR as an Ehd1 ortholog when comparing the receiver and GARP domains of *Ehd1* and of all rice and *Arabidopsis* B-type RR proteins found in the public databases (Fig. 3F). Other parts of Ehd1 than the receiver and GARP DNA-binding domains lacked significant homology with any database-registered protein. Furthermore, we found several pseudo-RRs and A-type RRs in the rice genome (Izawa et al. 2003; Murakami et al. 2003; data not shown). Therefore, *Ehd1* seems to have evolved originally as a B-type RR since the separation of monocot (rice) from dicot (*Arabidopsis*). It is very likely that the *Ehd1*-signaling cascade exists in rice but not in *Arabidopsis*.

Diurnal expression of Ehd1

In the T65 background, *Ehd1-gla* exhibited a diurnal expression pattern under SD (9L:15D) condition and a rapid damping of circadian oscillation in subsequent continuous darkness (Fig. 4A). Two peaks, one at 2 h before dawn and the other at 4 h after dawn, occurred under SD. The

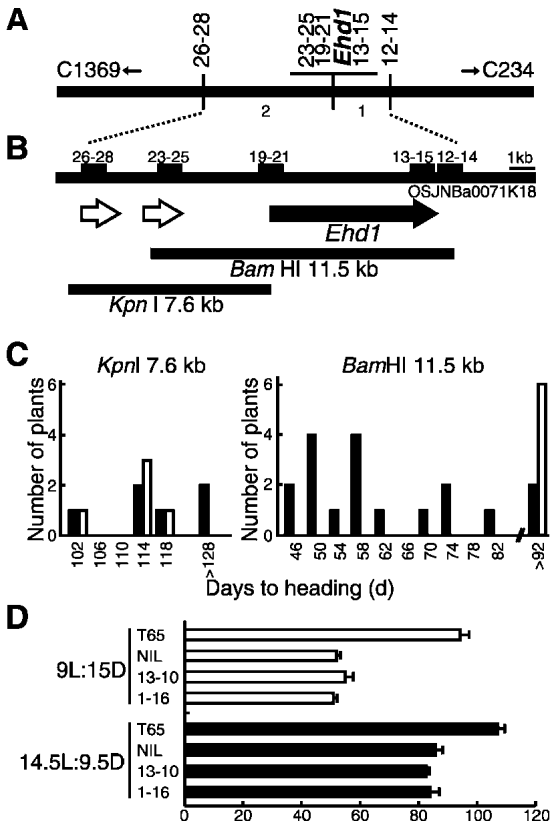

Figure 2. Map-based cloning of *Ehd1*. (*A*) High-resolution linkage map of *Ehd1* generated by using a mapping population of >2500 plants. *Ehd1* is located on the Clemson BAC clone OSJNBa0071K18 (GenBank accession no. AC027038). (*B*) Physical map of *Ehd1*. The CAPS markers (12–14, 13–15, 19–21, 23–25, and 26–28) used for the linkage analysis are indicated by bars. Genes predicted by the GENSCAN program (Burge and Karlin 1997) are shown by arrows. BamHI (11.5 kb) and KpnI (7.6 kb) genomic fragments from Kasalath were introduced into T65. (*C*) Days to heading of T_0 transformants under SD (10L:14D) condition. (Open bar) Empty vector; (solid bar) vector with the genome fragment. Only plants transformed with the 11.5-kb BamHI fragment showed promotion of flowering. (*D*) Days to heading of two independent T_2 lines homozygous for the *Ehd1-Kas* transgene (1–16, 13–10), NIL(*Ehd1-gla*) and T65 under SD (9L:15D) and LD (14.5L:9.5D) conditions.

second peak is probably an acute response to the light-on signals, because no such second peak was observed during continuous darkness. The damping of the rhythm in continuous darkness suggests that *Ehd1* is controlled also by circadian clocks. Therefore, both circadian clocks and acute responses to light may cause the diurnal rhythm of *Ehd1* mRNA levels. Under LD (15L:9D) conditions, the levels of *Ehd1-gla* mRNA were consistently low, whereas one peak was seen in subsequent continuous darkness 14 h after the onset of darkness, and a second peak occurred 15 h after the first peak (Fig. 4B). This suggests that *Ehd1* mRNA may be induced by a long-night treatment. In addition, the induction of *Ehd1* mRNA under SD strongly supports the idea that *Ehd1* functions as a floral inducer in the SD-promotion pathway.

mRNA levels of *ehd1-T65* did not show any clear diurnal or circadian patterns or any significant differences between SD and LD conditions, although irreproducible peaks were observed under LD (Supplementary Fig. 2). Whether *Ehd1-gla* mRNA oscillation is regulated by its own gene product in some feedback mechanism remains to be examined.

Ehd1 *promotes flowering by inducing* FT-like *genes*

FT-like genes are floral inducers of rice (Izawa et al. 2002; Kojima et al. 2002) and the possible targets of regulation by *Ehd1*. Therefore, we assessed the mRNA expression of *FT-like* genes in NIL(*Ehd1-gla*) and T65. After SD treatment, mRNA expression of *RFT1* and *Hd3a* increased in NIL(*Ehd1-gla*) and showed a diurnal expression with two peaks— one weak peak before dawn, and the other peak during daytime (Fig. 4C,D). In contrast, levels of expression were clearly lower in T65. Expression of *RFT1*, and *Hd3a* under LD was induced in neither T65 nor NIL(*Ehd1-gla*; data not shown). In addition, other *FT-like* gene expression was also examined by using a transgenic line that contains *Ehd1-Kas* in a T65 background (T65 + *Ehd1*), because at least nine *FT-like* genes exist in the rice genome (Izawa et al. 2002). In addition to *Hd3a* and *RFT1*, *FTL* and *FT-L9* were induced by *Ehd1* in the transgenic line (Fig. 4E). These results indicate that a novel two-component signaling cascade is integrated into the conserved photoperiod pathway in rice.

Other genes downstream of Ehd1

To further identify genes downstream of *Ehd1*, we performed a microarray analysis using custom 60-bp oligonucleotide arrays with 22,000 probes designed with full-length cDNA sequences of rice (Kikuchi et al. 2003). To minimize the effect of genetic background, we used T65 and T65 + *Ehd1* for the microarray analysis. We collected leaves from 1-month-old plants of T65 and T65 + *Ehd1* grown under SD, when floral transition could occur only in T6 5+ *Ehd1*, and prepared both total RNA and mRNA. Two independent color-swap experiments using both total RNA and mRNA provided us with 29 candidate genes, 21 up-regulated and 8 down-regulated, in T65 + *Ehd1* (Supplementary Table 1). Six of the 21 up-regulated genes were detected in common between the experiments using total RNA and mRNA. Subsequent RT–PCR analysis verified that at least 12 genes were induced and four were reduced by *Ehd1* upon floral transition (Fig. 5A–C; Table 1). We observed no difference in eight other genes by RT–PCR and could not detect any expression of the remaining five candidates. Some discrepancy between the results of microarray analysis and RT–PCR could be partly due to the low threshold of the log ratio, 0.25, when the candidate clones were selected in the microarray analysis.

Two of the twelve up-regulated genes are MIKC-type MADS box genes in rice. One of them is identical to *OsMADS14/RAP1B*, an *AP1* ortholog of rice, and the other is *OsMADS1*, which is related to the *SEP* family of

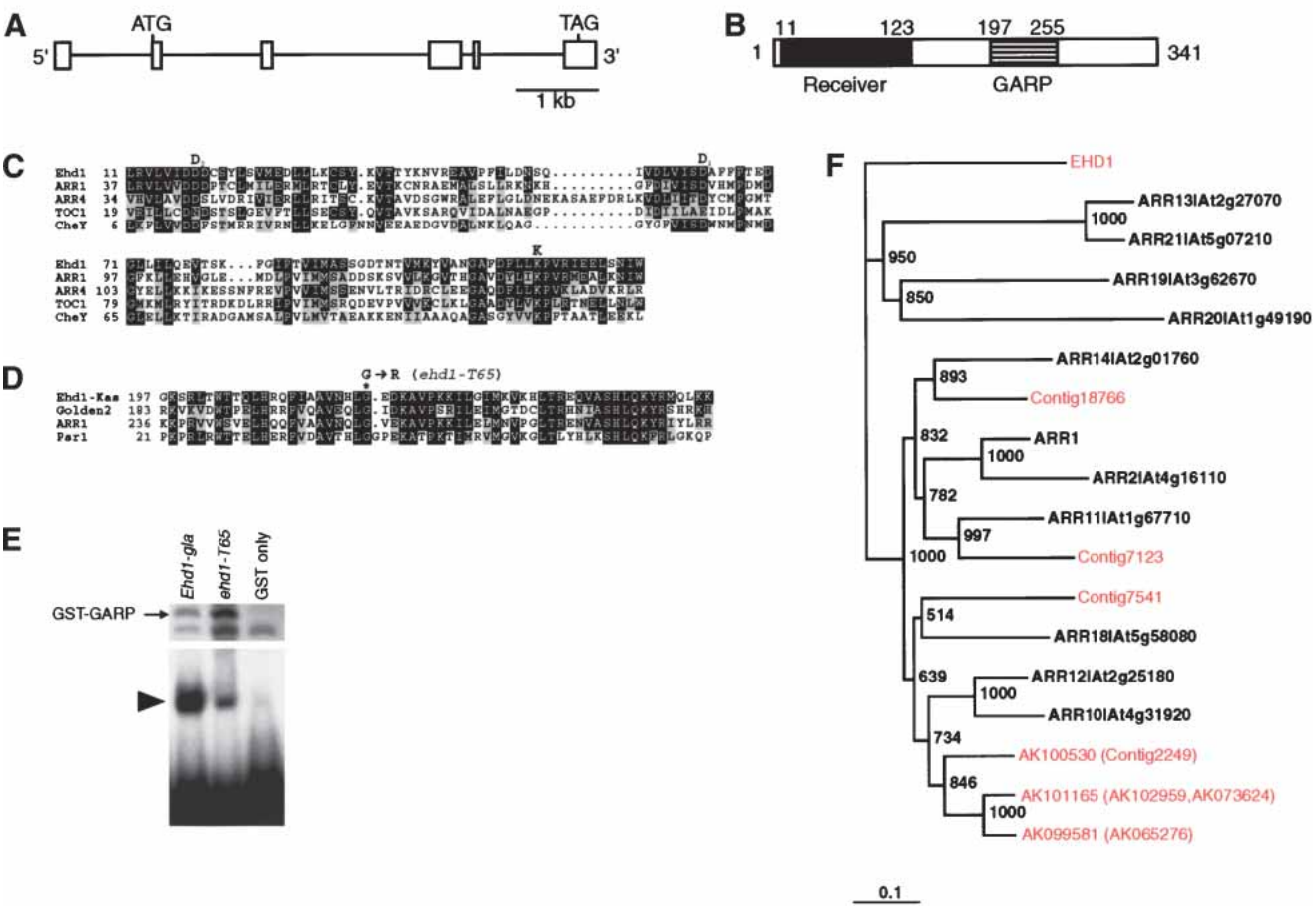

Figure 3. Structure of *Ehd1*. (*A*) Gene organization of *Ehd1*. White boxes indicate exons. (*B*) Protein structure of Ehd1. (*C*) Comparison of receiver domains of Ehd1-Kas and representative *Arabidopsis* response regulators ARR1 (B-type; GenBank accession no. T51246), ARR4 (A-type; BAA34726), TOC1 (Pseudo-RR; AAF86252), and CheY (BAA15698). Residues identical to those in Ehd1 are in black boxes; similar ones are in gray. Asp (D_1 and D_2) and Lys (K) residues crucial for the phosphorelay function are conserved in Ehd1. (*D*) Alignment of GARP domains of Ehd1-Kas, Golden2 (AAG32325), ARR1 (T51246), and Psr1 (AAD55941). The position of amino acid variation in T65 (G→R) is indicated by an asterisk. Sequences are highlighted as in *C*. (*E*) Comparison of the DNA-binding ability of the GARP domains of Ehd1-gla and ehd1-T65. Amounts of fusion proteins in *Escherichia coli* extracts are shown at the *top*. Extra bands in the GST–GARP lanes may be partly digested products. The same extracts were used for gel-mobility shift assay (*bottom*). The arrowhead indicates the band corresponding to the retarded probe. (*F*) Phylogenetic comparison of B-type RRs between rice and *Arabidopsis.* ARR clones with MIPS (Munich Information Center for Protein Sequences) codes correspond to *Arabidopsis* B-type RRs. The others are EHD1 and B-type RRs from rice database information. B-type RRs corresponding to rice full-length cDNAs are indicated by AK accession numbers. Sequences prefixed with Contig are derived from the 93-11 rice genome sequences of the Beijing Genomics Institute.

Arabidopsis (Fig. 5D) and is involved in flower development in rice (Jeon et al. 2000). These results are consistent with the view that *Ehd1* is a floral inducer. The relatively small number of genes regulated by *Ehd1* suggests that *Ehd1* signaling is specific to floral induction in rice. The biological functions of those downstream genes remain to be analyzed, because there are no apparent links to floral induction among downstream genes other than the OsMADS genes.

Identification of the two MADS box genes as genes downstream of *Ehd1* further led us to examine whether other MADS box genes possibly related to floral induction are affected by *Ehd1*. Phylogenetic analysis of MADS box genes using genomic and cDNA sequences of rice and *Arabidopsis* in public databases revealed that 12 rice MADS box genes belong to the *AP1/CAL/FUL*, *SEP*, and *SOC1* clades (Fig. 5D, in red). Note that no rice MADS box gene has been found in the *FLC* clade (Izawa et al. 2003). Among those MADS box genes, 10 correspond to full-length cDNA clones in the microarrays (AK clones in Fig. 5D). We confirmed that mRNA expression of eight MADS box clones other than *OsMADS14* and *OsMADS1* was not affected by *Ehd1* in the microarray analyses (data not shown). The remaining two MADS-box genes, *OsMADS15* (another *AP1* ortholog) and *OsMADS20*, were further examined by RT–PCR. The results indicate that *OsMADS15* was induced in T65 + *Ehd1*, whereas *OsMADS20* was slightly reduced

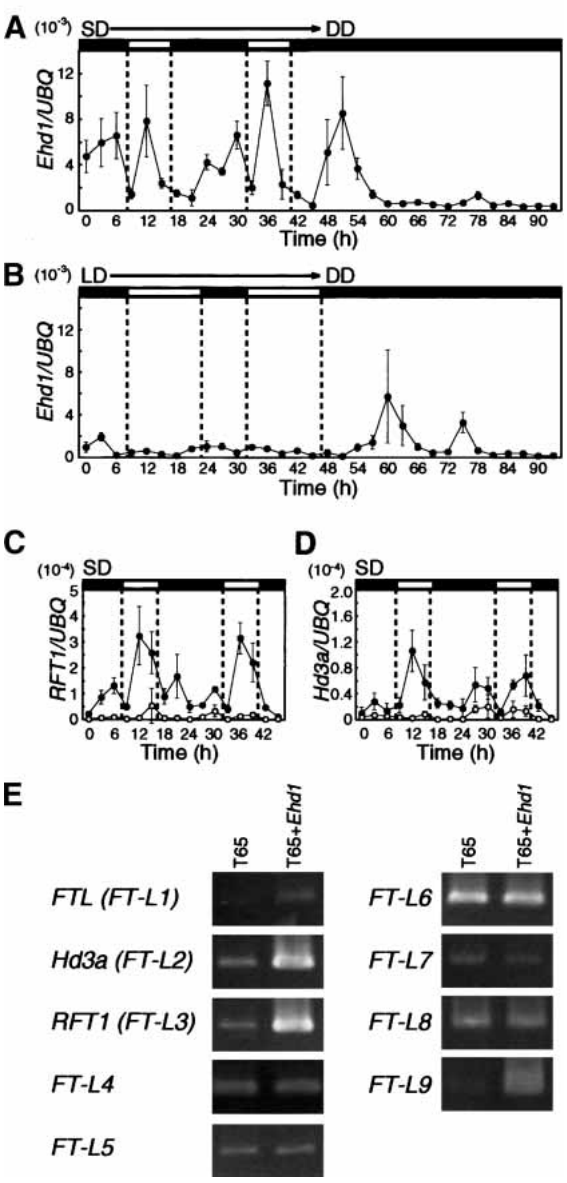

Figure 4. Expression analyses of *Ehd1*, *RFT1*, and *Hd3a*. (*A*,*B*) *Ehd1* mRNA expression in NIL(*Ehd1-gla*) under SD (*A*) and LD (*B*) condition. (*C*,*D*) Expression of *RFT1* (*C*) and *Hd3a* (*D*) in NIL(*Ehd1-gla*) (solid symbols) and T65 (open symbols) under SD condition. The ratios of the average expression levels of *Ehd1*, *RFT1*, and *Hd3a* to that of *UBQ* (ubiquitin) are plotted on the graphs. The average values were obtained from at least three real-time RT–PCR assays. Periods of light and darkness are indicated with white and black bars, respectively. (*E*) *FT-like* gene expression in T65 and T65 + *Ehd1* (Fig. 2D, lines 1–16).

(Fig. 5E). Therefore, three of the 12 rice MADS box genes—*OsMADS14*, *OsMADS1*, and *OsMADS15*—were induced by *Ehd1* upon floral transition.

Discussion

Hd1-*independent short-day promotion pathway in rice*

We have demonstrated that a B-type RR gene that may not have an ortholog in *Arabidopsis* plays an important role in photoperiodic control of flowering in the SD plant rice. We could conclude that *Ehd1* may not have an ortholog in *Arabidopsis*, as both rice and *Arabidopsis* genome sequences have been sequenced. However, it is possible that *Arabidopsis* B-type RR not-related to rice *Ehd1* might be involved in floral pathways. The fact that *Ehd1* can induce *FT-like* genes (Izawa et al. 2002; Kojima et al. 2002) in an *Hd1*-deficient background reveals that a novel two-component signaling cascade is integrated into the floral pathway. Floral regulation by *Ehd1* would be assigned as an SD-promotion pathway in rice. Genetic analyses indicated that *Ehd1-gla*, *Ehd1-Nip*, and *Ehd1-Kas* alleles were functional, and *ehd1-T65* was a recessive and loss-of-function allele. It is interesting that T65 still responds to photoperiod, although it has the loss-of-function alleles in both *Hd1* and *Ehd1*. There may be another unidentified photoperiodic floral pathway in rice, which is not genetically identified yet in T65. Alternatively, this may be due to the remained *Ehd1* activity of *ehd1-T65* with its low DNA-binding ability, although *ehd1-T65* mRNA expression pattern does not support this idea (Supplementary Fig. 2).

The expression pattern of *Ehd1* is clearly distinct from that of *Hd1*, which functions as a circadian clock mediator. *Hd1* mRNA is expressed during subjective night, and the expression patterns of *Hd1* mRNA are similar under both LD and SD conditions (Izawa et al. 2002). In contrast, *Ehd1* mRNA is induced only under SD conditions. This induction of *Ehd1* mRNA was observed in the presence of the defective allele of *Hd1*, although the expression of *Ehd1* is likely to be controlled by circadian clocks (Fig. 4A). Therefore, an unidentified circadian clock mediator other than *Hd1* may be involved in *Ehd1* mRNA expression.

Ehd1 *as a B-type response regulator*

It has been shown so far that plant two-component (or His-to-Asp phosphorelay) signaling cascades, which include histidine kinases (HKs), His-containing phosphotransfer proteins, and RRs, play important roles in phytohormone signaling in *Arabidopsis* (Stock et al. 2000; Lohrmann and Harter 2002). Ethylene receptors and cytokinin receptors encode HKs, although no molecular link between ethylene receptors and RRs was reported in *Arabidopsis* (Chang and Stadler 2001; Inoue et al. 2001). Recently, it was reported that an *Arabidopsis* HK, *CKI1*, which was believed to be a cytokinin receptor, plays a role in megagametogenesis (Pischke et al. 2002).

Phylogenetic analysis indicates that there are three major clades of RR in *Arabidopsis*, two clades of canonical RRs (A-type and B-type) and one atypical group (pseudo-RRs; Hwang et al. 2002). The rice *Ehd1* belongs to the B-type RRs, although none of the 12 B-type RRs of *Arabidopsis* seem to be orthologous to *Ehd1* (Fig. 3E). In *Arabidopsis*, *ARR1*, a B-type RR, functions in cytokinin signaling during *Arabidopsis* development (Sakai et al. 2001). In transient assays, the *ARR1*, *ARR2*, and *ARR10* B-type RRs are involved in cytokinin signaling and regu-

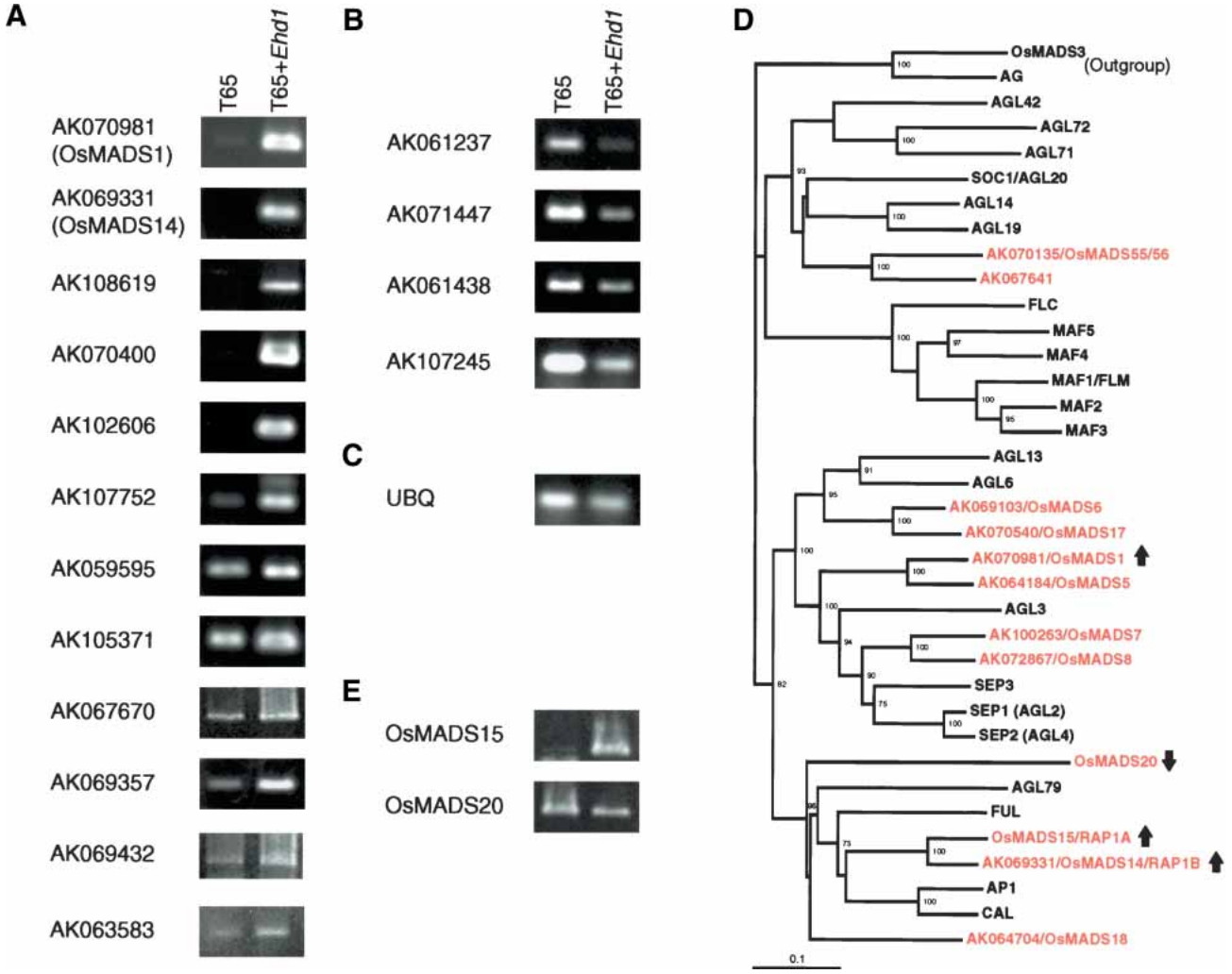

Figure 5. Genes downstream of *Ehd1* in RT–PCR assays. The same cDNA samples as used in Figure 4E were used as templates. (*A*) Clones up-regulated in T65 + *Ehd1*. (*B*) Clones down-regulated in T65 + *Ehd1*. (*C*) *UBQ* (ubiquitin) as a control. (*D*) Phylogenetic tree of MADS box genes in rice and *Arabidopsis*. All rice and *Arabidopsis* MADS box proteins belonging to AP1/CAL/FUL, SEP, and SOC1 clades in public databases plus AG and OsMADS3 as an outgroup are included in the tree. Accession numbers corresponding to full-length rice cDNAs are also shown. (*E*) RT–PCR of the remaining two rice MADS box genes, *OsMADS15* and *OsMADS20*.

late the mRNA expression of A-type RRs, such as *ARR6* (Hwang and Sheen 2001). Recent work has revealed that the *ARR11* B-type RR is also involved in cytokinin signaling (Imamura et al. 2003). In addition, a cytokinin-inducible A-type RR, *ARR4*, can interact directly with phytochrome B and may control stability of the Pfr form (Sweere et al. 2001). However, the biological functions of other *Arabidopsis* B-type RRs have not yet been identified.

In contrast, pseudo-RRs have been shown to regulate flowering time in *Arabidopsis*. *TOC1/APRR1*, an *Arabidopsis* pseudo-RR, is believed to be a plant circadian clock component (Matsushika et al. 2000; Strayer et al. 2000). It affects flowering time through the phase setting of *CO* expression (Yanovsky and Kay 2002). *TOC1/APRR1* belongs to a small gene family in *Arabidopsis* (Matsushika et al. 2000). Recently, at least five rice pseudo-RR genes orthologous to the *TOC1/APRR* family were analyzed to show circadian mRNA expression (Murakami et al. 2003). Pseudo-RRs may have lost their phosphorelay activity and are thus constitutively active because they lack highly conserved Asp residues. Because Ehd1 is a B-type RR with a canonical receiver domain and a functional GARP DNA-binding domain, it is likely to be evolutionarily distant from the pseudo-RR type and not to be a circadian clock component. Preferential expression of *Ehd1* mRNA only after SD treatment supports this idea. We examined the expression of a possible clock component in rice, *OsLHY* (Izawa et al. 2002), by a quantitative RT–PCR method, but did not find a significant difference in *OsLHY* expression by *Ehd1* (T. Izawa, K. Doi, A. Yoshimura, and M. Yano, unpubl.).

FT-like *and MADS box genes lie downstream of* Ehd1

The expression patterns of *Hd3a* and *RFT1* induced by *Ehd1* seem to parallel that of *Ehd1* mRNA expression

Table 1. *List of genes whose mRNA expression was affected by* Ehd1

Up- or down-regulated	GenBank accession no.	Corresponding rice genes	Most similar database protein excluding *Oryza sativa,* [species], score (bits), E value[a]
Up	AK059595		gi|15236625 polygalacturonase, putative [*Arabidopsis thaliana*], 569, le-161
Up	AK105371		gi|7433295 ω-6 desaturase [*Gossypium hirsutum*], 510, 1e-143
Up	AK069357		gi|15810385 unknown protein [*Arabidopsis thaliana*], 375, le-127
Up	AK070981	*OsMADS1* (S53306)	gi|3892652 putative MADS-domain transcription factor [*Zea mays*], 320, 2e-86
Up	AK069331	*OsMADS14* (AAF19047)/ *RAP1B* (BAA94342)	gi|29372748 putative MADS-domain transcription factor [*Zea mays*], 264, 2e-69
Up	AK067670	*LIRI* (Q03200)	gi|7448057 cytokinin-repressed protein CR9 [*Cucumis sativus*], 87, 4e-16
Up	AK069432		gi|30698182 senescence-associated protein [*Arabidopsis thaliana*], 78, 5e-13
Up	AK070400		gi|15241489 replication protein, putative [*Arabidopsis thaliana*], 75, 4e-12
Up	AK107752		gi|7488303 ribonucleoprotein homolog T4I9.1 [*Arabidopsis thaliana*], 46, 1e-04
Up	AK063583		gi|34530688 unnamed protein product [*Homo sapiens*], 41, 0.015
Up	AK102606		gi|37546363 hypothetical protein [*Homo sapiens*], 36, 1.9
Up	AK108619		No significant similarity found
Down	AK061438	Drought-induced S-like ribonuclease (AAL33776)	gi|20271131 S-like RNase [*Triticum aestivum*], 419, 1e-116
Down	AK071447		gi|8389329 (+)-delta-cadinene synthase [*Gossypium hirsutum*], 343, 5e-93
Down	AK061237		gi|21554074 putative acid phosphatase [*Arabidopsis thaliana*], 297, 3e-79
Down	AK107245		gi|15241275 ids4-related protein [*Arabidopsis thaliana*], 226, 9e-58

[a]The results of NCBI BlastX search against "nr" database (2003.10.16) excluding *Oryza sativa* clones.
Microarray clones verified by RT–PCR are summarized.

under SD (Fig. 4A,C,D). Because the GARP DNA-binding domain can specifically recognize only 5-bp nucleotides in vitro (Sakai et al. 2000) and *Ehd1* probably functions as a transcription factor, it is possible that *Ehd1* directly regulates the mRNA expression of these *FT-like* genes. Several possible binding sites of *Ehd1* in the *Hd3a* and *RFT1* promoter regions were found (data not shown), although the in vivo function of these candidate binding sites remains to be tested.

Previous studies have revealed that the expression of *FT-like* genes starts before dawn and continues until dusk in cultivars of rice that contain functional alleles of both *Hd1* and *Ehd1* (Izawa et al. 2002; Kojima et al. 2002). The expression of *FT-like* genes around dawn can be attributed to *Hd1*, but it does not explain the daytime expression of *FT-like* genes well (Izawa et al. 2002). However, the present study has provided a good explanation; *Ehd1* causes the expression of *FT-like* genes during daytime (Fig. 4). The *FT-like* genes are more highly expressed during daytime-induced peaks of *Ehd1* expression than the circadian peaks before down. In contrast, *Ehd1* mRNA exhibits bimodal expression patterns more clearly (Fig. 4C,D). Therefore, this daytime peak of *FT-like* genes may be affected by the external coincidence of light with *Ehd1* gene product.

On the other hand, we have identified three MADS box genes regulated downstream of *Ehd1*. Phylogenetic analysis tells that two of them, *OsMADS14* and *OsMADS15*, are orthologs of *Arabidopsis AP1*, and the other, *OsMADS1*, is related to the *Arabidopsis SEP* family (Fig. 5D). In *Arabidopsis* floral pathways, *AP1* lies downstream of floral integrators such as *FT*, *SOC1*, and *LEAFY* (Simpson and Dean 2002). Recently, wheat *VRN1* in the floral vernalization pathway was revealed to encode an *AP1* ortholog and to be induced by vernalization treatment (Yan et al. 2003). Meanwhile, *Arabidopsis SEP* genes are required for flower development (Pelaz et al. 2000), and the expression of *SEP1* and *SEP2* starts in stage-2 flower primordia upon floral transition (Flanagan and Ma 1994; Savidge et al. 1995). *OsMADS1* mRNA expression was detected in floral meristem regions upon floral transition (Chung et al. 1994). Furthermore, mutations in *OsMADS1* resulted in florets with elongated leafy paleas and lemmas, and overexpression of *OsMADS1* caused early flowering (Jeon et al. 2000). This evidence clearly indicates that *Ehd1* activates downstream genes such as *FT-like* genes and MADS box genes, which are tightly related to floral transition.

Two-component Ehd1 *signaling cascade in short-day promotion pathway*

Because a phytochrome deficiency derepresses mRNA expression of *FT-like* genes in rice (Izawa et al. 2002), the possibility that the *Ehd1* product is phosphorylated by phytochromes remains to be tested, although phosphorylation of B-type RRs has not been proved yet, even in *Arabidopsis*. Although no study has reported the HK ac-

tivity of a phytochrome in higher plants, HK activity has been detected in an ancestral phytochrome, *Cph1*, in cyanobacteria, and relevant RRs have been identified (Yeh et al. 1997). Alternatively, the acute expression of *Ehd1* in response to light-on signals suggests that *Ehd1* mRNA expression is regulated by phytochromes. It has been shown that cytokinin-inducible *ARR4* (an A-type RR; Brandstatter and Kieber 1998) can directly interact with phytochrome B in *Arabidopsis* (Sweere et al. 2001). Therefore, it is also possible that some A-type RRs transcriptionally regulated by *Ehd1* are involved in the control of the stability of Pfr phytochromes, and so, control *FT-like* genes in rice. However, downstream genes identified by microarray analysis alone do not support this idea at this moment.

Possible developmental or environmental signals mediated by *Ehd1* may include not only light signals, but also phytohormones such as cytokinins or ethylene, which may affect floral induction in some plant species (Bernier et al. 1993; Samach et al. 2000). All reported functions of B-type RRs were involved in cytokinin signaling in *Arabidopsis* (Hwang et al. 2002). However, profiles of genes downstream of *Ehd1* do not strongly support this idea, because all identified downstream genes seem not to be related to cytokinin signaling except for AK067670, which has some homology with the cytokinin-repressed *CR9* gene of *Cucumis sativus*, but is identical to the gene for lir1, a rice light-inducible protein (Table 1; Reimmann and Dudler 1993). Whether *Ehd1* can mediate cytokinin signaling is now under investigation. Whatever the signal is, *Ehd1* would lie in the latter part of the photoperiodic pathway of rice and function mainly as a floral inducer, because not many genes were found downstream of *Ehd1*. This floral inducer unique to rice will provide a clue to elucidating the diversity in the photoperiodic control of flowering of higher plants.

Materials and methods

Genetic analysis of Ehd1 *and* Hd1

For QTL analysis, 89 recombinant inbred (RI) lines derived from a cross between Nipponbare and T65 were planted at the experimental farm of Kyushu University. This experiment was conducted under natural field conditions. We analyzed the days-to-heading for 12 plants from each line to obtain the average of the line. The genotypes of the RI lines at the *Hd1* and *Ehd1* loci were determined by using PCR and cleaved amplified polymorphic (CAPS) markers.

For testing how *Ehd1* acts in photoperiodic flowering, the BC_6F_2 near-isogenic line NIL(*Ehd1-gla*), containing the functional allele of *Ehd1-gla* from *O. glaberrima*, was developed in the T65 background. T65 and NIL(*Ehd1-gla*) were grown under SD (10L:14D) and LD (15L:9D) conditions in the daylength control facility at Kyushu University. Three primers (P1, P2, and P3) were used for RT–PCR and genotyping (Fig. 1D,E,G).

Map-based cloning of Ehd1

The initial mapping of *Ehd1* by using a BC_3F_4 segregating population of 147 plants was reported previously (Doi and Yoshimura 1998). Progeny (>2500 plants) of heterozygotes of the initial mapping population were used for the high-resolution mapping. Linkage analysis using restriction-fragment-length polymorphism (RFLP) and CAPS markers revealed that *Ehd1* was restricted in a 16-kb genomic region on the BAC clone OSJNBa0071K18 (GenBank accession no. AC027038). To test the complementation of *Ehd1*, we cloned two Kasalath genomic fragments, the 11.5-kb BamHI fragment and the 7.6-kb KpnI fragment, into the pPZP2H-lac binary vector (Fuse et al. 2001) and introduced them into T65 by *Agrobacterium*-mediated transformation. The heading date of T_0 regenerated plants was examined under SD (10L:14D) condition after they were transplanted into soil in pots (Fig. 2C). Homozygous T_2 progeny derived from single-copy T_0 transformants were grown under SD (9L:15D) condition, and their heading dates were recorded. Only the 11.5-kb BamHI fragment could complement the *Ehd1* phenotype. The structure of the mRNA of *Ehd1-gla* was determined by rapid amplification of cDNA ends (RACE) with a Marathon cDNA amplification kit (Clontech). Gel-mobility shift assays of GARP domains were performed essentially as previously described (Sakai et al. 2000).

Quantitative RT–PCR analysis of gene expression

For SD samples, plants were grown for 3 wk under LD (15L:9D) condition and transferred to SD (9L:15D) condition for 1 wk. Then, leaves were harvested every 3 h for 4 d, 2 d under SD, followed by 2 d under continuous darkness. For LD samples, plants were grown under LD for 4 wk and the sampling was done for 4 d, 2 d under LD followed by 2 d under continuous darkness. Real-time quantitative RT–PCR analysis was performed as previously described (Kojima et al. 2002). cDNA corresponding to 100 ng 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 get average values of expression levels. The PCR conditions were 2 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, and 1 min, 30 sec at 60°C. For *Ehd1-gla* mRNA expression, the specific primers 5′-GCGCTTCTGATTTCC TGC-3′ and 5′-CGGAATATGTGCTGCCAG-3′ and the probe 5′-GTGAGGATCGAAGAGCTGAGCAACA-3′ were used. *RFT1* mRNA was quantified using the primer pair 5′-CGTCCATG GTGACCCAACA-3′ and 5′-CCGGGTCTACCATCACGAGT-3′ and the probe 5′-CGGTGGCAATGACATGAGGACGTTC -3′. *Hd3a* mRNA was quantified using primers and probes previously described (Kojima et al. 2002). For copy-number standards, quantified fragments of cloned cDNA were used.

Microarray analysis and RT–PCR

Agilent Technologies custom microarrays were hybridized according to the manufacturer's instructions. Because floral transition in T65 + *Ehd1* (Fig. 2D, lines 1–16) occurred ~25–30 d after sowing and *Ehd1* is expressed at daytime under SD condition, sample leaves of T65 and T65 + *Ehd1* were collected for RNA analysis at noon from 1-month-old plants grown under SD condition.

cDNA was synthesized from both total RNA and mRNA and labeled with the fluorescent dyes Cy5 and Cy3. Color-swap experiments were performed for both cDNA from total RNA and mRNA. Results were filtered to pick up candidate clones with an average log ratio >0.25 and a P value log ratio <0.01. The expression of candidate clones was verified by RT–PCR with specific primers under PCR conditions described in Supplementary Table 2. Independent RNA samples derived from different

plants grown under the same conditions were used for the RT–PCR verification.

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