Ehd4 Encodes a Novel and Oryza-Genus-Specific Regulator of Photoperiodic Flowering in Rice

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Abstract

Land plants have evolved increasingly complex regulatory modes of their flowering time (or heading date in crops). Rice (Oryza sativa L.) is a short-day plant that flowers more rapidly in short-day but delays under long-day conditions. Previous studies have shown that the CO-FT module initially identified in long-day plants (Arabidopsis) is evolutionarily conserved in short-day plants (Hd1-Hd3a in rice). However, in rice, there is a unique Ehd1-dependent flowering pathway that is Hd1-independent. Here, we report isolation and characterization of a positive regulator of Ehd1, Early heading date 4 (Ehd4). ehd4 mutants showed a never flowering phenotype under natural long-day conditions. Map-based cloning revealed that Ehd4 encodes a novel CCCH-type zinc finger protein, which is localized to the nucleus and is able to bind to nucleic acids in vitro and transactivate transcription in yeast, suggesting that it likely functions as a transcriptional regulator. Ehd4 expression is most active in young leaves with a diurnal expression pattern similar to that of Ehd1 under both short-day and long-day conditions. We show that Ehd4 up-regulates the expression of the ‘florigen’ genes Hd3a and RFT1 through Ehd1, but it acts independently of other known Ehd1 regulators. Strikingly, Ehd4 is highly conserved in the Oryza genus including wild and cultivated rice, but has no homologs in other species, suggesting that Ehd4 is originated along with the diversification of the Oryza genus from the grass family during evolution. We conclude that Ehd4 is a novel Oryza-genus-specific regulator of Ehd1, and it plays an essential role in photoperiodic control of flowering time in rice.

Introduction

Flowering is a profound transition from vegetative to reproductive development in plants, and is largely determined by genetic pathways that integrate endogenous and environmental signals [1]. Plants control flowering by perceiving their surroundings, such as day-length (photoperiod) and temperature that is synchronized with seasonal changes, in order to maximize their reproductive fitness [2]. Flowering time or heading date in crops is also a critical agronomic trait that determines the cropping season and regional adaptability of plants. Thus, control of flowering time has been extensively studied by plant breeders and scientists for more than 100 years [3].

Photoperiod control of flowering refers to the ability of plants to measure day-length and use it as an indicator to initiate flowering. Extensive studies in a model long-day plant (LDP), Arabidopsis thaliana, have revealed that light regulation of the GIGANTEA (GI)-CONSTANT (CO)-FLOWERING LOCUS T (FT) pathway is essential for integrating cellular signals from light signaling transduction and the circadian clock to promote flowering under long-day conditions (LDs) [4–6]. Phytochrome A (phyA), phytochrome B (phyB) and cryptochrome 2 (cry2) regulate FT expression by post-translationally regulating CO protein [7,8]. In addition, blue light promotes CO expression by stabilizing the FLAVIN-binding KELCH DOMAIN F BOX PROTEIN1 (FKF1)-GI protein complex [9,10]. CO, a zinc finger transcription factor, promotes FT expression under LDs by directly binding to its promoter [11,12]. FT, a small mobile protein functioning as the ‘florigen’, is synthesized in the phloem of leaves, and is then transported to the apical meristem where it initiates flowering by inducing the expression of the floral meristem identity genes, such as AP1 [13–15].

Rice (Oryza sativa L.) is an important source of calories for mankind and a model short-day plant (SDP) that flowers more rapidly in short-day conditions (SDs) but delays under LDs with a critical day-length response [16,17]. Previous studies have revealed that rice flowering is regulated both by a “SD-activation pathway” and a “LD-suppression pathway”. OsGIGANTEA (OsGI), Heading date 1 (Hd1) and Heading date 3a (Hd3a) have been identified as the counterpart of GI, CO and FT, respectively [18–20]. Hd1 executes dual function that promotes flowering by regulating Hd3a (the major SD ‘florigen’) expression under SDs, but suppresses it through unknown mechanisms under LDs [19,21,22]. However, the OsGI-Hd1-Hd3a pathway only plays a limited role in flowering.
time control in rice because there is a high degree of polymorphism in *Hd1* and non-functional alleles of *Hd1* are associated with only moderate phenotypic changes [23].

Rice has a unique, *Hd1*-independent flowering pathway that is mediated by *Early heading date 1* (*Ehd1*). *Ehd1* encodes a B-type response regulator that is highly conserved in cultivated rice, but has no homolog in Arabidopsis [23,24]. It has been shown that *Ehd1* positively regulates the expression of *Hd3a* and *RICE FLOWERING LOCUS T 1* (*RFT1*), the closest paralog of *Hd3a* that works as a LD ‘florigen’ [22,24,25]. Circumstantial evidence suggests that *Ehd1* is a critical convergence point of regulation by multiple signaling pathways. Among them, OsphyB inhibits flowering under both SDs and LDs by suppressing *Hd3a* expression through posttranslational modification of HD1 protein function and transcriptional suppression of *Ehd1* expression [25–27]. The OsphyB-mediated suppression of *Ehd1* is regulated by OsCOL4, which encodes a protein containing two B-box zinc finger domains and one CCT domain and it also acts as a constitutive suppressor of flowering in rice under both SD and LD conditions [26,27]. In addition, both *Ghd7* (Grain number, plant height and heading date 7), encoding a CCT domain protein [28], and *DTH8* (*Days to heading 8*), encoding a putative HAP3 subunit of the CCAAT-box-binding transcription factor, down-regulate *Ehd1* expression and delay flowering under LDs [29]. On the other hand, *Ehd1* expression is promoted by a number of positive regulators. Among them, *OsMADS51* encodes a type I MADS-box protein and induces *Ehd1* expression under SDs [30], whereas a rice homolog of Arabidopsis *SOC1* (*Suppressor of Overexpression of Constant1*), *OsMADS50*, was identified as a promoter of *Ehd1* expression under LDs [31]. Recently, it was shown that *Ehd1* expression could be independently up-regulated by *Early heading date 2/Rice Indeterminate 1* (*Oryza sativa Indeterminate 1*) (referred to as *Ehd2* hereafter) and *Early heading date 3* (*Ehd3*) under both SDs and LDs [32–34]. The former encodes a Cys2/His2-type zinc finger protein with high homology to maize *indeterminate1* [35], while the latter encodes a putative plant homeodomain (PHD) finger-containing protein. Notably, loss of function of *OsMADS50, Ehd2* and *Ehd4* showed a never-flowering phenotype under LDs [25,32,34,36]. Thus, it appears that *OsMADS50, Ehd2, Ehd3* and *Ehd1* may constitute a “LD-activation pathway” in rice. Although these studies have revealed much insight into the photoperiodic flowering of rice, the underlying molecular mechanisms are still not well understood.

Here we report the identification of *Early heading date 4* (*Ehd4*) using a mutagenesis approach and its positional cloning. *Ehd4* encodes a novel CGCH (C-X2–C-X–C-X–H) type zinc finger protein and it acts as a critical regulator promoting flowering under both SDs and LDs, particularly under LDs. Mutation in *Ehd4* causes a never-flowering phenotype under natural long-day conditions (NLDs). *Ehd4* protein is localized to the nucleus and it has nucleic acid-binding and transcriptional activation properties, consistent with a plausible function as a transcription factor. We show that *Ehd4* promotes flowering by up-regulating the expression of *Hd3a* and *RFT1* through stimulation of *Ehd1* expression. Interestingly, *Ehd4* is highly conserved in the *Oryza* genus and it has no homologs in other plant species. Thus, our findings identified a novel, highly conserved rice-specific regulator of flowering time.

**Results**

**Characterization of the late flowering mutant *ehd4***

In an effort to isolate genes that are essential for promoting flowering time in rice, we generated a large T-DNA population in a day-length neutral, early flowering variety Kita-ake (*O. sativa ssp. japonica*). Kita-ake (*Kit*) has been widely used in rice transformation experiments because of its short life cycle. Kit flowers about two months after germination under both SDs (10 h light/14 h dark) and LDs (14.5 h light/9.5 h dark) conditions in the controlled growth chamber, as well as under natural long-day field conditions (NLDs) in Beijing (39°54′N, 116°23′E), North China (Figure 1A and 1B). To understand the day-length neutral nature of Kita-ake, we cloned ten genes reported to have significant effect on flowering time in rice, including seven genes that promote flowering (*Ehd1* to 3, *OsMADS50, OsMADS51, Hd3a* and *RFT1*) and three genes that suppress flowering under LDs (*Hd6, Hd4* and *Hd7*), and compared them with the corresponding genes in Nipponbare (Nip), a *japonica* variety that is sensitive to day-length. Those flowering-promoting genes are identical in Kit and Nip varieties, except *OsMADS51* that contains one amino acid variation (Figure S1). In contrast, Kit has an immature stop in *Ghd7* and a 36-bp insertion and two amino acid changes in *Hd1* (Figure S1). Although there is no difference in *Hd6* sequences between Kit and Nip, both of them have an early stop compared with the allele of the *indica* variety Kasalash (Figure S1), which delays flowering in Nip background under LDs [37,38]. Therefore, complete or partial loss of function of those three genes in Kit could at least partially explain its insensitivity to day-length.

We screened our T-DNA population in the NLDs and identified a mutant that failed to flower during the 160 days of growing season (from late April to early October, 2006), whereas the wild-type (WT) Kit flowered 55 days after germination (Figure 1A and 1B). We were able to produce seeds by moving this mutant plant to a controlled SDs. Plants from an F2 population derived from a cross of the mutant and WT segregated in field conditions into three categories based on their flowering time (days after germination): 36.9±1.8, 70.8±1.8 and never flowering mutants in a ratio of 1:2.1 (χ²[1:2.1]=0.415<χ²[0.05,2]=5.99, n=200). This result indicates that the mutation is semidominant and is controlled by a single gene. We named this locus *Ehd4* (*Early heading date 4*). Compared with WT, *ehd4* delayed flowering time by
49 d and 106 d under SDs and LDs, respectively (Figure 1B). Consistent with field observations, flowering time of the heterozygotes was also delayed under both SDs and LDs (Figure 1B). Notably, ehd4 had a similar leaf emergence rate to WT under both SDs and LDs (Figure 1C), indicating that the late flowering phenotype is not caused by retardation in growth rate. The mature ehd4 plants were taller, producing more but smaller seeds. The fertility of ehd4 plants was similar to that of WT (Figure 1D–1H).

To test whether the delayed flowering phenotype is genetic background-dependent, we introduced the ehd4 locus into Nip by backcrossing five times, followed by selfing. The ehd4-NIP plants (BC5F3) delayed flowering by 23 d under SDs compared to the WT (Kita-ake) under both SDs and LDs (n = 8). Arrow indicates the flowering time of WT plants. (D) Panicle morphology of WT and ehd4 plants. (E) to (H) Comparisons of grain number per panicle (E), 1000-grain weight (F), plant height (G) and fertility (H) between WT and ehd4 plants. Values are means ± s.d. (standard deviations) (n = 15). **Significant at 1% level; n.s., not significant.

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**Figure 1. Characterization of Ehd4.** (A) Never-flowering phenotype of ehd4 mutants in field (Top). WT, Kita-ake wild-type plants (Bottom). (B) Flowering time of ehd4, heterozygote (HETE) and WT plants under different day length conditions in Kita-ake (day-length neutral) and Nipponbare (day-length sensitive) backgrounds (n = 12). ND, natural-day; SD, short-day; LD, long-day. (C) ehd4 plants had the same leaf emergence rate as WT (Kita-ake) under both SDs and LDs (n = 8). Arrow indicates the flowering time of WT plants. (D) Panicle morphology of WT and ehd4 plants. (E) to (H) Comparisons of grain number per panicle (E), 1000-grain weight (F), plant height (G) and fertility (H) between WT and ehd4 plants. Values are means ± s.d. (standard deviations) (n = 15). **Significant at 1% level; n.s., not significant.

**Molecular cloning of Ehd4.**

Flowering time control in rice is regulated by the interaction of multiple QTLs and the environments. Generally, flowering time of F2 population derived from japonica×indica displays a normal distribution pattern. Therefore, we crossed ehd4 with 93-11, an indica variety with an available genome sequence [39], and generated a BC1F2 population for mapping the ehd4 locus by backcrossing the F1 with 93-11. The ehd4 locus was initially mapped to the short arm of chromosome 3 (Figure 2A). Using 871 extremely late flowering plants from approximately 25,000 BC1F2 plants grown in Hainan Island (18°45’N, 110°02’E, average day length 11 hours), South China, during the winter of 2008, we further delimited Ehd4 to a 103 kb region, between the markers EF-4 and EF-5 (Figure 2B). This region contains 16 annotated ORFs (http://rapdb.dna.affrc.go.jp) (Figure 2C). Sequencing of the genomic DNA of all these genes revealed that there is a single nucleotide substitution (G to A) in the first exon of LOC_Os03g02160, which is predicted to encode a CCCH-Type zinc finger protein. The nucleotide change creates a premature stop codon at the very beginning of the predicted coding region (Figure 2D). Genomic sequence of this gene is identical between Kit and Nip (Figure S1). Quantitative real-time PCR (qRT-PCR) assay showed comparable expression of LOC_Os03g02160 in wild type, heterozygote and ehd4 mutant plants (Figure S2). Transgenic plants carrying the full-length cDNA of LOC_Os03g02160, driven by the maize Ubiquitin-1 promoter, fully complemented the ehd4 phenotype under both SDs and LDs. Further, cDNA driven by its native promoter (2.7 kb upstream from ATG) also partially rescued the ehd4 phenotype. The phenotypes of these transgenic lines (days to flowering) appeared to correlate with the expression level of Ehd4 (Figure 2E and Figure S2). Thus, we concluded that the LOC_Os03g02160 locus corresponds to Ehd4.

**Expression of Ehd4 is constitutive and diurnal.**

We examined the expression levels of Ehd4 in various tissues and at different stages of leaf development (Figure 3A) by using qRT-PCR. Ehd4 transcripts were detected in all tissues examined, but the highest expression was found in emerging young leaves and the lowest level in fully expanded leaves (Figure 3B). Histochemical staining of transgenic plants carrying the GUS reporter gene driven by the Ehd4 promoter indicated that GUS was expressed in all tissues examined and was most abundant in the vascular tissue and apical meristem (Figure 3C–3I). The expression of Ehd4 showed a diurnal expression pattern in leaves. It accumulates after dusk, reaching a peak at dawn, and damping rapidly thereafter under both SDs and LDs (Figure 3J). Moreover, Ehd4 was expressed constantly during the vegetative growth from the second week to the 10th week after germination (Figure 3K).

**EHD4 may act as a transcriptional regulator.**

In higher plants, CCCH-type zinc finger proteins have been shown to regulate gene expression by binding to DNA or RNA.
molecules in the nucleus [40–42]. We fused Ehd4 with GFP and transiently expressed the EHD4-GFP fusion protein in rice leaf protoplasts. EHD4-GFP was exclusively co-localized with the OsMADS3-mCherry fusion protein (a nuclear marker) in the nucleus (Figure 4A–4C), indicating that EHD4 functions in the nucleus. We further fused EHD4 and its various deletions with the GAL4 DNA binding domain and investigated if EHD4 has transcriptional activation activity in yeast. Full-length wild type EHD4 and an EHD4 variant with only the CCCH motif removed were able to activate the reporter gene expression (Figure 4D). Further deletion of the C terminal region resulted in a dramatic reduction of the activation activity, whereas deletion of both the N-terminal and CCCH motif only had mild effects (Figure 4D). These observations suggest that the activation domain is located in the middle region close to the C-terminal of EHD4. In addition, a nucleic acid binding assay demonstrated that the C-terminal region, but not the N-terminal region, can bind to both double- and single-stranded calf thymus DNA and ribohomopolymers in vitro, and that removal of the CCCH motif from the C-terminal abolished the binding activity (Figure 4E). These results strongly support the notion that EHD4 likely functions as a transcriptional activator and that the CCCH motif is essential for its nucleic acid binding activity.

**Figure 2. Map-based cloning of Ehd4.** (A) Location of the Ehd4 locus on rice chromosome 3. (B) High-resolution linkage map of Ehd4. (C) Candidate genes on BAC OSJNBb0005F16. (D) Structure of the Ehd4 gene. Lines, black and white boxes represent introns, exons and untranslated regions, respectively. The base change from G to A creates an early stop codon (Asterisk). (E) Complementation of ehd4. Ehd4 was driven by either the native promoter (pEhd4::Ehd4) or the maize Ubiquitin-1 promoter (pUbi::Ehd4). T2 plants of two pEhd4::Ehd4 lines (#26 and #34) and two pUbi::Ehd4 lines (#18 and #24) were measured (n = 10). All plants were grown under both SD and LD conditions. doi:10.1371/journal.pgen.1003281.g002

**Figure 3. Expression pattern of Ehd4.** (A) 30-d-old wild-type plants (Kita-ake) grown under SDs were used for quantitative RT-PCR. DL1, newly emerging leaf; DL2, expanding leaf; DL3, fully expended leaf; ASA, around the shoot apex. (B) Ehd4 transcript levels in various organs (means ± s.d., n = 3). (C) to (I) GUS staining of various organs in pEhd4::GUS transgenic plants. (C) Root; (D) Floret; (E) Stem; (F) to (H) Transverse sections of stem, immature leaf and sheath, respectively; (I) Longitudinal section of the shoot apical meristem (SAM). Arrow indicates phloem in (F) and (G) and SAM in (I). (J) and (K) Rhythmic and developmental expression of Ehd4. The rice Ubiquitin-1 (UBQ) gene was used as the internal control. Values are shown as mean ± s.d of three independent experiments and two biological replicates. The open and filled bars at the bottom represent the light and dark periods, respectively. s.d: standard deviations. doi:10.1371/journal.pgen.1003281.g003

**Ehd4 regulates expression of the “florigen” genes through Ehd1**

Photoperiodic induction of the floral transition in rice requires transcriptional activation of Hd3a and RFT1, the two ‘florigen’ genes, in leaves [20–22]. The diurnal expression pattern of Ehd4 implies that it could be involved in photoperiodic control of flowering. To test this, we examined mRNA abundance of Hd3a and RFT1 in ehd4 and WT plants by qRT-PCR. The expression levels of Hd3a and RFT1 were undetectable in ehd4 under both SDs and LDs at all-time points examined during the 48 h period (Figure 5A–5D). Subsequently, expression of the downstream genes OsMADS1, OsMADS14 and OsMADS15 (three floral meristem identity genes; [22,25]) was severely impaired in the ehd4 mutants (Figure S3). Hd1 and Ehd1 are known to regulate Hd3a and RFT1 [19,24]. To investigate whether the activation of ‘florigen’ genes by Ehd4 is mediated by Hd1 and/or Ehd1, we compared their mRNA levels between ehd4 and WT plants. Strikingly, expression of Ehd1, but not Hd1, was abolished in ehd4 mutants, indicating that Ehd4 is essential for Ehd1 expression (Figure 5E–5H). Ehd4 has a diurnal expression pattern similar to that of Ehd1, typically peaking at dawn (Compare Figure 3J with Figure 5E and 5F). Next, we examined whether Ehd4 affects the expression of other known regulators of Ehd1. To our surprise, the transcription levels of five positive regulators ([Ehd2, Ehd3, OsMADS50, OsGFI and OsMADS31]) and four negative regulators of Ehd1 (OsphyB, OsCOLA, DTH8 and
Figure 4. EHD4 is a nuclear protein with intrinsic transcriptional activation and nucleic acid binding activities. (A) Subcellular localization of EHD4-GFP fusion protein. (B) The nuclear marker MAD3-mCherry fusion protein. (C) Merged image of (A) and (B) under bright field. Scale bar = 10 μm in (A) to (C). (D) Transactivation assays of EHD4 and its deletion derivatives in the yeast GAL4 system. Full length EHD4 and several deletion derivatives of EHD4 (pEhd4-Δ, pEhd4-N and pEhd4-CΔ) were used in assays. The empty vector (BD-MCS) and BD-DST (Δ6) were used as negative and positive control, respectively. Transformants were dropped onto SD/Trypt- and SD/Histidine- plates to allow growth of 48 hours before taking pictures. Values in β-galactosidase activity are means of three independent experiments. Bars stand for standard deviations. BD, DNA-binding domain of GAL4. (E) The CCCH motif is essential for binding to nucleic acids. C terminal, N terminal or C terminal without CCCH motif of EHD4 was expressed in E.coli and purified for binding assays. Deletion of the CCCH motif abolished the binding to ribonucleoproteins and both double- and single-stranded calf thymus DNA.

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Ehd4 Regulates Rice Photoperiodic Flowering

Ehd4 is unique and highly conserved in rice

Ehd4 is a single copy gene in the rice genome. It is predicted to code for a polypeptide of 832 amino acids long, which contains a CCCH (C-X7-C-X5-C-X3-H)-type zinc finger motif at the C-terminus (Figure S9). A blast search (http://www.ncbi.nlm.nih.gov/) found that EHD4 has no clear homologs in other plant or animal species. Thus it appears that Ehd4 represents a unique regulator of flowering time in rice.

To investigate the evolutionary history of the gene in rice, we analyzed the Ehd4 sequences from 86 rice accessions with wide geographic distribution and diverse genetic backgrounds, including 32 wild rice species (O. rufipogon and O. nivara) and 54 cultivated rice (Table S2; [48]). Ehd4 appears to be highly conserved across these accessions (share >99.2% or higher amino acid sequence identities) (Table S2). Sequence analysis identified 25 haplotypes among these accessions. Strikingly, 21 haplotypes were identified in 32 wild rice accessions (O. rufipogon and O. nivara) but only 8 haplotypes were identified in 54 cultivated rice accessions analyzed in this study. The dramatic reduction in genetic diversity at this locus suggests that Ehd4 might subject to bottleneck effect [49]. Notably, 4 haplotypes (Hap2, 3, 6 and 7) are shared in cultivated and wild rice (Figure 4A, Table S2), and among them, Hap2 and Hap3 together account 25% of the 32 wild rice and 85% of the through Ehd1. This notion was also supported by the observation that over-expression of Ehd1 fully rescued the late flowering phenotype of ehd4 under SDs (Figure 7).

Since EHD4 has a transcriptional activation and nucleic acid binding activity and it promotes Ehd1 expression, we next carried out a yeast one-hybrid assay and a transient transcription assay [43,44] to test whether Ehd1 is a direct downstream target of EHD4. However, only OsLFL1 [45], but not EHD4, was able to interact with the Ehd1 promoter (Figure S5), indicating that Ehd1 is likely an indirect target of Ehd4. In addition, yeast three-hybrid assay [46] also failed to detect a direct binding of EHD4 to the Ehd1 mRNA (Figure S6). Moreover, neither EHD2 nor EHD3, directly binds to the Ehd1 promoter (Figure S5). Our yeast two-hybrid assay showed that there was no direct interaction among the EHD2, EHD3 and EHD4 proteins (Figure S7). Together, these results suggest that Ehd2, Ehd3 and Ehd4 likely act through distinct pathways to promote the expression of Ehd1.

Transcriptome analysis of ehd4 plants

To further reveal the molecular basis of the flowering phenotype of ehd4, we performed a transcriptome analysis of ehd4 and wild-type plants using RNA-seq to identify genes downstream of Ehd4. RNA samples were extracted from the penultimate leaves (collected at dawn) of 30 ehd4 and WT plants (Kita-ake) grown under LDs. We obtained 2.5 M tags and found a total of 256 genes altered in expression with an estimated false-discovery rate of 0.1% and the absolute value of log2Ratio at 3.15 under the Bayesian model (Table S1; [47]). We found that the transcript numbers of Hd3a, RFT1, Ehd1, OsMADS1, OsMADS14 and OsMADS15 reduced dramatically in ehd4 plants (Table S1), consistent with the qRT-PCR results (Figure 5A-S and Figure S3). Our qRT-PCR analysis with other four genes (with a log2 Ratio of −11.73, −10.33, −5.80 and −3.15, respectively) also further confirmed the reliability of the RNA-seq results (Figure S8). Notably, we found that among the genes down-regulated in ehd4, 25 of them are known or putative transcription factors, including MADS box, Zinc finger, MYB, SBP and B3 proteins (Table S1). These genes could be potential candidates involved in the Ehd4-Ehd1-Hd3a/RFT1 pathway to regulate photoperiodic flowering in rice.

Ghd7) were not significantly affected in ehd4 (Figure 6A and 6B). These observations suggest that Ehd4 functions upstream of Ehd1, but largely independent of other known regulators of Ehd1. Consistent with this, down regulation of Ehd1, Hd3a and RFT1 in ehd4 was also seen in the Nipponbare background and constantly seen at different stages during plant development (Figure 6B and Figure S4).

To investigate whether Ehd4 expression is regulated by other flowering genes, we examined the expression of Ehd4 in osphyb, ehd2, ehd3, osmads50 and osmads51 mutants and near-isogenic lines (NILs) which carrying a deficient H1i, Ghd7 or DTH8 alleles. Notably, we detected no significant differences of Ehd4 expression in these mutants or NILs, as compared to their corresponding WT plants (Figure 6C). In addition, no significant change of Ehd4 expression was seen in NILs deficient in Ehd1 or Hd3a either (Figure 6C). These results suggest that Ehd4 acts independent of other Ehd1 regulators we examined. Together, these observations suggest that Ehd4 regulates the expression of Hd3a and RFT1...
Discussion

In this study, we have uncovered Ehd4, which codes for a CCCH-type zinc finger protein essential for promoting flowering under both SD and LD conditions in rice, irrespective of genetic backgrounds. We demonstrated that Ehd4 promotes flowering by positively regulating the expression of Hd3a and RFT1 through Ehd1 but independent of other known important Ehd1 regulators. We further showed that the late-flowering phenotype of ehd4 is more profound in Kita-ake (Kit) (a day-length neutral variety) than in Nipponbare (Nip) (a day-length sensitive variety) under SDs, but ehd4 plants flowered eventually in Kit (164 days after germination) but not in Nip under LDs (Figure 1B). It is known that Hd1 acts to promote flowering under SDs but delay flowering under LDs [19]. We found that Hd1 has a 36-bp insertion and two SNPs in Kit compared to Nip (Figure S1), implying that in Kit, the promoting role under SDs and repressing role under LDs of Hd1 may be impaired. This may at least partially explains why the late-flowering phenotype of ehd4-Nip is less severe under SDs but more severe under LDs. In addition, we found that Kit carries a truncated allele of Ghd7, a repressor of Ehd1 under LDs, whereas Nip carries a partially functional Ghd7 allele (Figure S1; [28]). Therefore, the never-flowering phenotype of ehd4 in Nip under LDs could be due to the repressive effect of Hd1 and Ghd7. Strikingly, even in the absence of the functional Hd1 and Ghd7 alleles in Kit, ehd4 alone delayed flowering time by three folds under LDs (Figure 1B), suggesting that Ehd4 plays a major role in promoting flowering in rice, particularly under LDs.

Previous studies revealed that rice Ehd1 is a critical convergence point of flowering time regulation by multiple signaling pathways and that Ehd1 acts independently of Hd1. Ehd1 encodes a B-type response regulator that is highly conserved in cultivated rice, but it has no homolog in Arabidopsis [23,24]. Up to date, 12 genes have been shown to regulate Ehd1 expression, including 5 positive regulators (Ehd2, Ehd3, OsGI, OsMADS50, and OsMADS54) and 7 negative regulators (SE5, OsphyB, Ghd7, DTH8, OsLFL1, OsCOL4, and OsMADS56). We demonstrated that Ehd4 promotes flowering by positively regulating the expression of Hd3a and RFT1 through Ehd1, but independently of these known Ehd1 regulators.

It is also of interest to note that the majority of Ehd1 regulators uncovered thus far are nuclear proteins and many of them act as transcriptional regulators, including GHD7, DTH8, OsMAD50, OsMAD51, EHD2, EHD3, OsLFL1, OsMAD56 and OsCOL4 [26,28–34,36,45,50]. Map-based cloning revealed that Ehd4 encodes a novel CCCH-type zinc finger protein also localized to the nucleus. The CCCH-type zinc finger protein family is defined as a group of proteins containing 1–6 copies of the canonical C-X-C-X-H motif (C-X6–14-C-X4–5-C-X3-H, where X is any amino acid) [51]. This type of proteins has been found in organisms ranging from human to yeast and many of them have been shown to have either an RNA binding function involved in RNA processing or DNA binding activity [40–42]. There are at least 68 CCCH-type genes in Arabidopsis and 67 in rice, respectively [52]. However, only a few plant CCCH proteins

Figure S10). This result suggests that Hap_3 of Ehd4 is functionally more potent in promoting flowering than Hap_2. As another test of this notion, we introduced the Hap_3 allele from Kita-ake into 93-11 by backcrossing five times, followed by selfing. Strikingly, the NIL Ehd4Hap_3 plants (BC3F2) flowered earlier by 19 d under NLDs compared to the parental 93-11 plants (Figure 8D). Together, these results suggest that the functional differences of Ehd4 haplotypes might play a role in geographic adaptation of cultivated rice.
have been functionally characterized. EHD4 is the first CCCH-type protein found to regulate photoperiodic flowering. We found that EHD4 is capable of binding to nucleic acids in vitro and transactivate transcription in yeast, suggesting that it likely functions as a transcription factor. Further, our transcriptome analysis revealed that a significant portion of Ehd4-regulated downstream genes are also transcription factors, including MADS box, Zinc finger, MYB, SBP and B3 proteins (Table S1). These findings together suggest that transcriptional regulation plays a critical role in photoperiodic regulation of flowering in rice. However, despite we have demonstrated that EHD4 has double-stranded DNA and ribohomopolymer binding activity and transactivation activity in yeast, we have not been able to identify the direct target genes of EHD4 in this study. It is also possible that EHD4 may bind to RNA molecules and degrades transcripts of unknown Ehd1 repressors.

Figure 6. Quantitative RT–PCR analysis of representative flowering-related genes and Ehd4 in various flowering-time mutants or their NILs (near-isogenic lines) and corresponding WTs under SDs and LDs. (A) Transcript level of Ehd2, Ehd3, OsMADS50, OsGl, OsMADS51, OsphyB, OsCOL4 and DTH8 in WT (Kita-ake) and ehd4 plants. (B) Transcript level of Gh7d, Hd3a, RFT1, Ehd1 and Hd1 in WT (Nipponbare) and ehd4-Nip plants. (C) Transcript level of Ehd4 in various flowering-time mutants or their NILs (near-isogenic lines) and corresponding WTs. Dongjin and the osphyb mutant [26]; Tohoku IL9 and the ehd2 mutant [34]; Tohoku IL9 and the ehd3 mutant [36]; Dongjin and the osmads50 mutant [31]; Dongjin and the osmads51 mutant [30]; Nipponbare and a NIL carrying a non-functional Hd1 allele [19]; Asominori and a NIL carrying a non-functional DTH8 allele [29]; A NIL carrying a functional Gh7d allele and a NIL carrying a non-functional in the Shanyou 63 background [28]. Taichun 65 carrying a non-functional Ehd1 allele and a NIL carrying a functional Ehd1 allele [24]; Nipponbare carrying a partially functional Hd3a allele and a NIL carrying a functional Hd3a allele [20]; Penultimate leaves were harvested around reported peak expression level of each gene during the 24 hrs photoperiod - at dawn for OsphyB, OsCOL4, Ehd1, Ehd2, Hd3a, RFT1 and Ehd4, 3 h after dawn for Gh7d, 8 h after dawn for Ehd3, OsMADS50, OsMADS51 and DTH8 and immediately after dusk for OsGl and Hd1 from 28 d-old (SDs) and 35 d-old (LDs) plants. The rice Ubiquitin-1 (UBQ) gene was used as the internal control. Values are shown as mean±s.d (standard deviations) of three independent experiments and two biological replicates.

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Further studies are required to elucidate the biochemical function of EHD4 and its functional relationship with other nuclear regulators of flowering time.

Rice is known as a short day plant. However, cultivated rice (O. sativa) is grown widely in Asia, with a northern limit of nearly 53°N in northern Asia (Northern provinces of China and Korea, where natural day length during rice cultivation is nearly 15 hours light; [53]), whereas O. rufipogon, a wild rice that is the most relative ancestor of O. sativa, is mainly distributed at tropical latitudes with a northern limit about 28°N [54]. The northward expansion of cultivated rice into higher latitudes must be accompanied by human selection of the flowering time trait during rice domestication and breeding, to secure a harvest before cold weather approaches. Strikingly, Ehd4 has no obvious homologs in other plant species including Arabidopsis, maize and sorghum, suggesting that Ehd4 originated along with the diversification of the Oryza genus from the grass family during evolution. Amino acid sequence comparison of EHD4 showed identities at 99.2% or higher among a core collection of rice germplasm with wide geographic distribution and diverse genetic backgrounds, including wild rice species ([48]; Table S2). Interestingly, we found two major haplotypes of Ehd4, Hap_2 (the major haplotype in indica) and Hap_3 (the major haplotype in japonica) and that Hap_3 is functional more potent in promoting flowering under NLDs. Since indica rice is known to distribute mostly in lower latitude and elevation zones (between the latitude 3°S-35°N), while japonica varieties are mostly distributed in higher latitude and elevation zones (between the latitude 15°N-53°N), our findings suggest that Ehd4 may have contributed to the northward expansion and regional adaptation of cultivated rice into higher latitudes.

Materials and Methods

Plant material and growth conditions

The ehd4 mutant was initially identified from a tissue culture-derived population of rice cv Kita-ake (japonica) under natural-day conditions in a paddy field in Beijing (39°54′N, 116°23′E), China (2006). To generate ehd4-rip plants, the mutant locus was introgressed into Nipponbare (japonica) background by crossing and backcrossing for five generations (BC5), where ehd4 mutant is the donor parent and Nipponbare is the recurrent parent, by using marker assisted selection (MAS).

Plants were grown in controlled-growth chambers (Conviron) under SDs (10 h light at 30°C/14 h dark at 25°C) or LDs (14.5 h light at 30°C/9.5 h dark at 25°C) with a relative humidity of ~70%. The light intensity was ~800 μmol m−2 s−1.

Map-based cloning

To map the ehd4 locus, the mutant was crossed with the indica cv 93-11 and then the F1 plants were backcrossed with 93-11 to produce a BC1F2 population. We used two DNA pools generated from 15 BC1F2 late-flowering and 15 normal plants, respectively, for rough mapping. For fine mapping, 871 never-flowering plants segregated in the BC1F2 population were used.

Vector construction and plant transformation

For the complementary test, the Ehd4 full-length cDNA driven by its native (2.7-kb) or the maize Ubiquitin-1 (Ubi) promoter were cloned into the binary vector pCAMBIA1390 by using In-Fusion Advantage PCR Cloning Kits (Clontech) to create pUbi::Ehd4 and pUbi::Ehd4, respectively. The 2.7-kb long promoter was also cloned into pCAMBIA-1303.1 to create pUbi::Ehd4-GUS. The resultant plasmids were transformed into the Agrobacterium tumefaciens strain EHA105 and then introduced into ehd4 (for complementary test) or Kita-ake WT plants (pUbi::Ehd4-GUS). At least 15 transgenic events were produced for each construct.

Subcellular localization of EHD4

GFP was fused to the C-terminus of EHD4 under the control of the 35S CaMV promoter in the pA7 vector. The EHD4-GFP fusion and the nucleus marker OsMADS3-mCherry were expressed in E. coli.

Transactivation activity assay

Transactivation activity assay was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Plasmids containing GAL4 DNA binding domain fused with EHD4 deletions were transformed into the yeast strain AH109. The substrate chlorophenol red-β-D-galactopyranoside (CPRG; Roche Biochemicals) was used to measure the β-galactosidase activity according to the Yeast Protocols Handbook (Clontech).

In vitro nucleic acid binding assay

EHD4 deletions were cloned into the pMAL-c2x (NEB) and expressed in E. coli. 0.5 mg purified protein was incubated with 20 μL of poly rG, poly rC, or poly rU attached to agarose beads (Sigma) in 500 μL of RHPA binding buffer (10 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100, 0.1 M NaCl at various concentrations) with 1 mg/mL heparin. After incubation at 4°C for 10 min, the beads were washed five times in the RHPA buffer and then boiled in the SDS loading buffer. Binding of fusion proteins to RNA or DNA was confirmed by gel blot using anti-MBP antibodies (NEB).

Yeast one-hybrid assay

Yeast one-hybrid assay was performed according to the method described in [43]. To generate GAD-EHD4, GAD-EHD2, GAD-EHD3 and GAD-OrsLF1L1, their full-length cDNAs were cloned into pJG4-5 vector. To generate the Ehd1p::LacZ reporter gene, a 3.2 kb fragment of Ehd1 promoter (including the 5′-UTR) was cloned into the binary vector pCAMBIA1390 by using In-Fusion Advantage PCR Cloning Kits (Clontech) to create pUbi::Ehd4 and pUbi::Ehd4, respectively. The 2.7-kb long promoter was also cloned into pCAMBIA-1303.1 to create pUbi::Ehd4-GUS. The resultant plasmids were transformed into the Agrobacterium tumefaciens strain EHA105 and then introduced into ehd4 (for complementary test) or Kita-ake WT plants (pUbi::Ehd4-GUS). At least 15 transgenic events were produced for each construct.
Figure 8. Natural variations in the \textit{Ehd4} coding region among rice germplasm core collection. (A) Haplotype network of the \textit{Ehd4} alleles in 86 rice accessions. Haplotype frequencies are proportional to the area of the circles. The proportion of wild rice and two cultivated subgroups (\textit{indica} and \textit{japonica}) in each haplotype is represented by different colors. (B) The polymorphic nucleotides of Hap\textsubscript{2} and Hap\textsubscript{3} of \textit{Ehd4} gene in the core collection. The number on the top shows the position of nucleotide polymorphisms in the coding region starting from the ATG start codon. (C) Geographic distribution of the cultivated rice accessions belonging to Hap\textsubscript{2} and Hap\textsubscript{3}. (D) Flowering time of transgenic plants carrying two major
amplified from Nipponbare genomic DNA and inserted into the corresponding sites of the reporter plasmid pLacZI2m. Plasmids were co-transformed into the yeast strain EGY48. Transformants were grown onto SD/Trp-/-Ura plates for 48 hours and then transferred onto X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) plates for blue color development.

Yeast three-hybrid assay
Yeast three-hybrid assay was performed as described in [46]. Full-length Ehd4 cDNA was cloned into pACTII vector to generate GAD-EHD4. A series DNA fragments (about 140 bp) that transcribing Ehd1 mRNA sequence were cloned into pMS2-1 vector. Plasmids were co-transformed into the yeast strain YBZ-1. Transformants were grown on plates containing selective media (SD/Ura-/Leu-/His-0, 2 or 8 mM 3-aminoatrazole) for 48 hours before assay.

Bioluminescence assays
Isolation of rice leaf protoplast and PEG-mediated transfection were performed as described [55]. The reporter construct pGreen-Ehd1p-LUC and effector plasmids (pEGAD-MycEHD4, EHD2, EHD3 or OsLFL1) were co-transformed into protoplasts. After transformation, the protoplasts were incubated in darkness for 12–16 h. Bioluminescence assay was performed according to the method described in [44].

Quantitative real-time RT-PCR
Total RNA was extracted using the RNeasy Plant Mini Kit (QiAGEN). For quantitative real-time RT-PCR, the first-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QiAGEN) and then PCR was performed using gene-specific primers and SYBR Premix ExTaq reagent (Takara) with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. PCR reactions were carried out in triplicate for each sample from two independent biological replicates and the rice Ubiquitin-1 gene was used as the internal control.

RNA–seq (next-generation sequencing of RNA)
We used the Illumina HiSeq 2000 Genome Analyzer to get tags with CATG site, in which the adapter sequences are 2*100 bp. With Illumina’s digital gene expression assay, we obtained 11.7 million sequence tags per sample. After removing low quality reads and low sequence tags, we generated 8.9 million perfect match tags (76.08%) for each sample. Mismatches of not more than one base were allowed in the alignment and we obtained 8.9 million perfect match tags (76.08%) for each sample. Initially, we determine 27020 genes of significant differences in expression between the groups of wild-type and mutants by a Student’s t-test. With a dedicated Bayesian model, we found 256 transcripts of differential expression with an estimated false-discovery rate of 0.1% and the average value of log2Ratio is more than 3.15.

All primers used in this study are listed in Table S3.

Accession numbers
Data deposition: The Ehd4 sequence reported in this paper has been deposited in the GenBank database accession no. JQ828863 (cDNA).

Supporting Information

Figure S1 Alignment of the open reading frames of eleven flowering related genes between Kita-ake and Nipponbare. Kasa, Kasalash. SM, synonymous mutations; Asterisks represent the premature stop codon.

Figure S2 Transcript levels of Ehd4 in WT (Kita-ake), heterozygote (HETE), ehd4 and transgenic plants. Penultimate leaves were harvested at dawn from 28 d-old (SDs) and 35 d-old (LDs) plants. The rice Ubiquitin-1 (UBQ) gene was used as the internal control. Values are shown as mean±s.d (standard deviations) of three independent experiments and two biological replicates.

Figure S3 Transcript levels of OsMADS14, OsMADS15 and OsMADS17 in WT (Kita-ake) and ehd4 plants. Penultimate leaves were harvested around reported peak expression level of each gene during the 24 hrs photoperiod - at dawn from 28 d-old (SDs) and 35 d-old (LDs) plants. The rice Ubiquitin-1 (UBQ) gene was used as the internal control. Values are shown as mean±s.d (standard deviations) of three independent experiments and two biological replicates.

Figure S4 Developmental expression pattern of Ehd1, Hd3a and RFT1 in WT (Kita-ake) and ehd4 plants under SDs (A, C and E) and LDs (B, D and F). The rice Ubiquitin-1 (UBQ) gene was used as the internal control in the quantitative RT-PCR analysis. Values are shown as mean±s.d (standard deviations) of three independent experiments and two biological replicates.

Figure S5 Yeast One-Hybrid and Bioluminescence assays. (A) GAD-LFL1 (positive control; [45]), but not GAD-EHD2, GAD-EHD3, GAD-EHD4 or GAD itself (negative control), strongly activate expression of the Lat52 reporter genes driven by the Ehd1 promoter (3.2 kb upstream of the ATG start codon) in yeast one-hybrid assay. (B) Structure of the vector used for transient expression. 35S, 35S CaMV promoter; REN, renilla luciferase; 35S mini, 35S CaMV minimum promoter; LUC, luciferase gene; 35S term, 35S CaMV terminator. (C) Relative reporter activity (LUC/REN) in rice protoplasts. Bioluminescence assays showing that expression of Ehd1::LUC reporter was not induced by EHD4, EHD2, EHD3 or GAD (empty vector) itself but strongly repressed by LFL1 (positive control; [45]) in rice leaf protoplasts. The relative LUC activities normalized to the REN activity are shown (LUC/REN, n = 3).

Figure S6 Yeast Three-Hybrid assay. (A) Genomic structure of the Ehd1 locus. Regions used for assays in (B) are underlined and numbered in order. (B) Yeast Three-Hybrid assays showing that EHD4 did not interact with any part of Ehd1 mRNA. The plasmid pIIIA/IRE-MS2 expressing 5' IRE-MS2 3' hybrid RNA from the yeast RNAseP promoter and the plasmid pAD-IRP expressing the rabbit Iron Regulatory Protein fused to the Gal 4 Activation Domain was used as the positive control [46] and the corresponding empty vectors were used as the negative control.
**Figure S7** Yeast Two-Hybrid Assay. Yeast two-hybrid assays showing that EHD2, EHD3 and EHD4 did not interact with each other. BD-OsMADS50 and AD-OsMADS56 were used as the positive control [50] and the empty vector were used as the negative control. ND, not determined.

**Figure S8** qRT-PCR confirmation of RNA-seq results. Four genes with reduced expression in ehd4 as determined by RNA-seq, were chosen for qRT-PCR assay. Independent penultimate leaves of 28-d-old plants grown under LDs were collected at dawn. The rice Ubiquitin-1 (UBQ) gene was used as the internal control. Values are shown as mean±s.d (standard deviations) of three independent experiments.

**Figure S9** Alignment of the CCCH motif of EHD4 with the zinc fingers from other CCCH-type proteins. Zinc fingers are from rice OsLIC (Os06g49080) and OsDOS (Os01g09620), Arabidopsis FES1 (At2g33385), HUA1 (NP_187874), PEI (S22126), SOM-NUS (At1g03790), SZF1 (At3g55980) and SZF2 (At2g10140), Cotton GhZFP1 (AY387895), C. elegans MEX-1 (U81043), PIE-1 (POSTECH, Korea) for mutants (osphyb, osmads50, and osmads51), Dr. Qiya Zhang (HZAU, China) for NILs (Ghd7), and Dr. Roger I. Pennell (Ceres Inc., USA) and Dr. Song Ge (IBCAS, China) for their constructive suggestions and critical reading of the manuscript.

**Table S1** Transcript analysis of ehd4 plants. Genes with expression level changes of at least 8 fold (ehd4/Wild Type) were listed.

**Table S2** Summary of the ehd4 allele types in cultivated and wild rice.

**Table S3** Primers used in this study.

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