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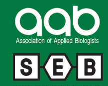

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OsRE1 interacts with OsRIP1 to regulate rice heading date by finely modulating *Ehd1* expression

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Keywords: rice (*Oryza sativa*), *Ehd1*, heading date, *OsRE1*, *OsRIP1*.

Summary

Heading date is a key agronomic trait affecting crop yield. In rice, Early heading date 1 (Ehd1) is an important B-type response regulator in determination of heading date. Although many regulatory factors of *Ehd1* expression have been functionally characterized, the direct regulators of *Ehd1* largely remain to be identified. Here, we identified a new regulator of *Ehd1*, OsRE1, that directly binds to the A-box motif in the *Ehd1* promoter. *Osre1* confers an early heading phenotype due to elevated expression levels of *Ehd1*. OsRE1 is a nucleus-localized bZIP transcription factor with a diurnal rhythmic expression pattern. Furthermore, we identified an OsRE1-interacting protein, OsRIP1, and demonstrated that OsRIP1 can repress the transcript expression of *Ehd1* in an OsRE1-dependent manner. Our genetic data showed that OsRE1 and OsRIP1 may function upstream of *Ehd1* in regulating heading date. Together, our results suggest that OsRE1 functions cooperatively with OsRIP1 to regulate heading date through finely modulating the expression of *Ehd1*. In addition, OsRE1 and OsRIP1 are two minor heading date regulators, which are more desirable for fine-tuning heading date to improve rice regional adaptability.

Introduction

Rice (*Oryza sativa*) is one of the most important cereal crops in the world by serving as a staple food for more than half of the world population (Foley *et al*., 2011). Heading date (flowering time) is an essential biological trait in rice that influences regional adaptation and grain production (Izawa, 2007). Heading date is determined by multiple external and internal signals, including photoperiod, temperature and hormones (Luan *et al*., 2009). Photoperiod is the most important environmental factor affecting heading date in rice (Song *et al*., 2015), a typical short-day plant. Several flowering pathways affected by photoperiod were identified (Sun *et al*., 2014). The *HEADING DATE1* (*Hd1*) pathway is conserved both in rice (*OsGI-Hd1-Hd3a*) and in *Arabidopsis thaliana* (*GI-CO-FT*) (Shrestha *et al*., 2014). *OsGI* (*GIGANTEA*) and *Hd1* in rice are homologs of *GI* and *CO* (*CONSTANS*) in Arabidopsis, respectively (Hayama *et al*., 2002; Yano *et al*., 2000). Unlike *CO* which merely promotes flowering in Arabidopsis, *Hd1* plays a dual role in rice by promoting flowering under short-day (SD) conditions and repressing flowering under long-day (LD) conditions (Hayama *et al*., 2003; Zhang *et al*., 2017). The *EARLY HEADING DATE1* (*Ehd1*) pathway is unique to rice and was reported to function independent of *OsGI-Hd1-Hd3a* in controlling heading date (Doi *et al*., 2004). Both pathways target the florigens HEADING DATE 3a (Hd3a) and RICE FLOWERING LOCUS T1 (RFT1), which have high amino acid sequence similarity with FLOWERING LOCUS T (FT), the florigen in Arabidopsis (Kojima *et al*., 2002; Komiya *et al*., 2008; Tamaki *et al*., 2007). Notably, Hd1 and PLANT HEIGHT AND HEADING DATE 7 (Ghd7, a strong flowering repressor in *Ehd1* pathway) were reported to form complex, which specifically bind to a *cis*-regulatory region in *Ehd1* promoter and repressed its expression, suggesting that the OsGI-Hd1-Hd3a pathway likely integrates into the *Ehd1* pathway under LD conditions (Nemoto *et al*., 2016).

Ehd1 encodes a B-type response regulator (Doi *et al*., 2004). Many proteins regulate *Ehd1* expression. OsMADS50, OsMADS51, RICE INDETERMINATE 1 (RID1)/EARLY HEADING DATE 2 (Ehd2), EARLY HEADING DATE 3 (Ehd3), EARLY HEADING DATE 4 (Ehd4), Hd3a BINDING REPRESSOR FACTOR 1 (HBF1) and SDG723/OsTrx1/OsSET33 INTERACTION PROTEIN 1 (SIP1) function as positive regulators of *Ehd1* (Brambilla *et al*., 2017; Gao *et al*., 2013; Jiang *et al*., 2018; Kim *et al*., 2007; Lee *et al*., 2004; Matsubara *et al*., 2011; Matsubara *et al*., 2008; Wu *et al*., 2008). OsCOL4, GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7 (Ghd7), DAYS TO HEADING ON CHROMOSOME 8 (DTH8/Ghd8/OsHAP3H), OsCOL9, OsCOL10, OsCOL13, OsHAPL1, OsMFT1 and OsIDS1 function as repressors of *Ehd1* (Lee *et al*., 2010; Lee *et al*., 2014; Liu *et al*., 2016; Sheng *et al*., 2016; Song *et al*., 2018; Tan *et al*., 2016; Wei *et al*., 2010; Xue *et al*., 2008; Zhu *et al*., 2017). Of them, only HBF1 and SIP1 bind to the *Ehd1* promoter directly (Brambilla *et al*., 2017; Jiang *et al*., 2018). Although many regulators of *Ehd1* have been identified, direct regulators are rarely reported, and the mechanisms by which *Ehd1* is directly regulated have remained elusive.

The basic leucine (Leu) zipper (bZIP) transcription factor (TF) family is one of the largest and most diverse TF families in plants

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(Dröge-Laser *et al*., 2018). bZIP proteins are named by their conserved bZIP domain, which contains 60-80 amino acids (Landschulz *et al*., 1988). There are 89 bZIP-encoding genes in rice genome (Nijhawan *et al*., 2008). bZIP proteins participate in multiple biological processes including pathogen defence, response to abiotic stress, phytohormone activity, flowering, senescence, seed development and germination (E *et al*., 2014). In Arabidopsis, bZIP protein FD interacts with FT in the shoot apical meristem (SAM) to promote flowering (Abe *et al*., 2005; Wigge *et al*., 2005). In rice, the homolog of FD, OsFD and an additional bZIP protein HBF1(Hd3a BINDING REPRESSOR FACTOR 1) interact with Hd3a/RFT1 in the SAM and regulate *Ehd1* expression in leaves (Brambilla *et al*., 2017). Another bZIP protein, OsABF1 (ABA RESPONSIVE ELEMENT BINDING FACTOR 1), mediates drought-derived signals to regulate heading date through indirect suppression of *Ehd1* (Zhang *et al*., 2016).

BBX proteins (B-box-containing proteins) are a class of zinc finger proteins containing one or two B-box domains (Borden, 1998; Torok and Etkin, 2001). 30 *BBX* genes (*OsBBXs*) were identified in rice through genome-wide surveys (Huang *et al*., 2012). OsBBX proteins are divided into five subfamilies according to the number of B-box domains and presence or absence of a CCT domain (Griffiths *et al*., 2003). BBX proteins play important roles in plant growth and development, such as seedling photomorphogenesis, photoperiodic regulation of flowering, shade avoidance and response to biotic or abiotic stress (Cheng and Wang, 2005; Crocco *et al*., 2015; Gangappa *et al*., 2013; Kumagai *et al*., 2008). The BBX proteins Hd1 and OsCO3, known as CO homologs in rice, have been reported to regulate heading date (Kim *et al*., 2008; Yano *et al*., 2000). OsBBX14, encoding a BBX TF with two B-boxes and no CCT motif, delays heading date by acting as an indirect repressor of *Ehd1* (Bai *et al*., 2016). Although BBX protein is reported to affect flowering time, its physiological significance and its regulatory mechanism have remained elusive.

Here, we identified a direct regulator of *Ehd1*, OsRE1, a bZIP transcription factor that was localized in the nucleus and exhibited rhythmical expression pattern. *Osre1* showed earlier heading date with elevated expression levels of *Ehd1*. Biochemical assays showed that OsRE1 physically interacts with OsRE1-INTERACTING PROTEIN 1 (OsRIP1), a BBX transcription factor. *Osrip1* also shows early heading with elevated expression levels of *Ehd1*. Biochemical and genetic evidence showed that OsRIP1 cannot directly bind to but facilitate OsRE1 to bind to the *Ehd1* promoter to repress the *Ehd1* expression. Furthermore, our genetic data suggest that OsRE1 and OsRIP1 may function upstream of *Ehd1*. Our combined molecular, biochemical and genetic data support this notion that OsRE1 forms a transcriptional inhibition complex with OsRIP1 and these factors act cooperatively in regulating heading date through finely repressing the transcript expression of *Ehd1* in rice.

Results

OsRE1 could directly bind to the *Ehd1* promoter *in vivo* and *in vitro*

To identify direct regulators of *Ehd1*, yeast one-hybrid (Y1H) library screening assay was performed using an *Ehd1* promoter fragment. LOC_Os01g07880, a bZIP transcription factor, was selected as a potential candidate. We designated it regulator of *Ehd1*, OsRE1. To further confirm the precise binding region for OsRE1, the *Ehd1* promoter was divided into five fragments for Y1H assay. OsRE1 specifically bound to P2, P3 and P5 fragments but not P1 and P4 fragments (Figure 1a). Interestingly, we found that P2, P3 and P5 fragments constantly contain an A-box motif (TACGTA; 638 bp in the *Ehd1* promoter), a preferential binding site for bZIP proteins (Hartmann *et al*., 2005; Izawa *et al*., 1994). To confirm the interaction *in vivo*, we performed a chromatin immunoprecipitation (ChIP)-qPCR assay using Flag-OsRE1 transgenic plants. Region S4 (−692 to −537 bp) containing the A-box motif was clearly enriched in the presence of the fused protein (Figure 1b), indicating that OsRE1 could bind to the *Ehd1* promoter *in vivo*.

We next performed electrophoretic mobility shift assay (EMSA) using a biotin-labelled *Ehd1* promoter fragment (−656 to −615 bp) containing the A-box motif (−638 to −633 bp). As shown in Figure 1c, OsRE1 bound to the probe containing the A-box motif (lane 2) and the shift band was suppressed when a competitor probe was used (lanes 3–5), whereas a labelled probe containing two mutated nucleotides did not bind (lane 6). Collectively, these results indicated that OsRE1 can directly bind to the *Ehd1* promoter.

OsRE1 is a minor-effect negative regulator of *Ehd1*

To investigate whether *OsRE1* is involved in determination of heading date in rice, we generated the *Osre1* mutant in rice cultivar Nipponbare using the CRISPR-*Cas9* technology. We selected two independent homozygous transgenic lines with different editing (+1 bp and −2 bp) for further study (Figure 2a). Compared with Nipponbare, the *Osre1* mutant exhibited a 3.7-d early heading phenotype under NLD conditions in Beijing (Figure 2b, c). Agronomic traits including plant height, panicle length and grains per panicle were differentially reduced compared to WT, but 1000-grain weight is not changed (Table S1). Given that OsRE1 could bind to the *Ehd1* promoter, we examined whether the expression level of *Ehd1* was affected by OsRE1. As shown in Figure 2d, the expression level of *Ehd1* was higher in the *Osre1* mutant than in WT under LD conditions. Together, these results suggested that OsRE1 is a minor-effect negative regulator of *Ehd1*.

Subcellular localization of OsRE1 and expression pattern of *OsRE1*

To determine the subcellular localization of OsRE1, we fused it with green fluorescent protein (GFP) under the control of the cauliflower mosaic virus CaMV *35S* promoter (*35S: OsRE1-GFP*) and used mCherry-fused D53 as a nuclear marker (Zhou *et al*., 2013). As shown in Figure 3a, OsRE1-GFP fluorescence signals are well merged with the D53-mCherry signals, suggesting that OsRE1 is a nuclear protein.

To examine the temporal and spatial expression pattern of *OsRE1*, qRT-PCR was performed to analyse the expression levels of *OsRE1* in different tissues including root, stem, sheath, leaves and panicles. *OsRE1* was expressed in all tissues examined, with a relatively high expression in leaves and panicles (Figure 3b).

We then compared the diurnal expression patterns of *OsRE1* under both LD (14-h light/10-h darkness) and SD (10-h light/14-h darkness) conditions. Similar diurnal expression patterns of *OsRE1* were evidently observed under both LD and SD conditions. Expression levels rose quickly to a peak at dawn and then decreased rapidly to the lowest point at dusk (Figure 3c). After Nipponbare grown in LD conditions was transferred to continuous light or darkness, rhythmic expression patterns of *OsRE1* were destroyed (Figure 3d).

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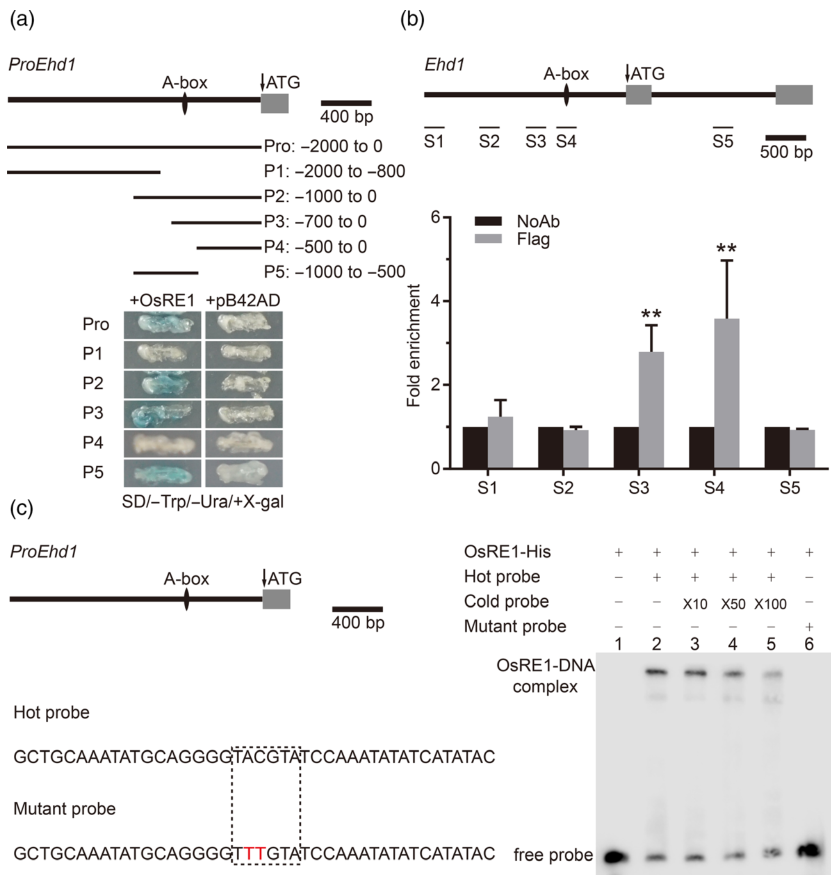

Figure 1 OsRE1 directly binds to the promoter of *Ehd1*. (a) Y1H assay showed that OsRE1 can bind to the *Ehd1* promoter fragments containing A-box motif (−638 bp). The promoter of *Ehd1* was divided into five fragments (P1 to P5). An empty vector was used as the negative control. Yeast was grown on Synthetic Drop-Out (SD) medium containing X-gal and lacking Trp/Ura. (b) ChIP-qPCR assays showing *in vivo* binding of OsRE1 to the *Ehd1* promoter. Cross-linked chromatin samples were extracted from Flag-OsRE1 transgenic plants and then precipitated with anti-Flag antibody. NoAb (No antibody) served as a negative control. Values are means ± SD; $n = 3$. Asterisks indicate significantly different values (**, $P < 0.01$). (c) EMSA showed that OsRE1 binds to the A-box motif of the *Ehd1* promoter. The hot probe was a biotin-labelled fragment of the *Ehd1* promoter containing the A-box motif, and the cold probe was a nonlabelled competitive probe. The mutant probe was the labelled hot probe sequence with two nucleotides mutated. His-tagged OsRE1 was purified, and 2 μg protein was used. [Colour figure can be viewed at wileyonlinelibrary.com]

OsRE1 physically interacts with OsRIP1 *in vitro* and *in vivo*

To further investigate the function of OsRE1 in determination of heading date, we performed yeast two-hybrid (Y2H) screening using pGBKT7-OsRE1 as bait. We screened a cDNA library prepared from leaves and identified a positive clone, namely *OsRE1-INTERACTING PROTEIN 1* (*OsRIP1*, *LOC_Os04g45690*). Blast search showed that OsRIP1 was annotated as the BBX transcription factor, OsBBX13 (Huang *et al*., 2012), but its function remains unknown. Similar to the typical BBX proteins, OsRIP1 contains two conserved B-box domains (Gangappa and Botto, 2014; Figure S1, Table S3). We further isolated coding sequence for full-length OsRIP1 and confirmed its interaction with OsRE1 (Figure 4a). To determine the region of both OsRE1 and OsRIP1 for their interaction, we constructed a deletion series of OsRIP1 based on domain prediction (http://pfam.xfam.org/) and examined the interaction with full-length OsRE1 and *vice versa*. We identified the bZIP domain of OsRE1 and the second B-box domain of OsRIP1 as the necessary domains for the interaction between OsRE1 and OsRIP1 (Figure S2a, b).

An *in vitro* pull-down assay confirmed that MBP-OsRIP1, but not free MBP, pulled down the OsRE1-His (Figure 4b). To visualize the interaction between OsRE1 and OsRIP1, we performed a biomolecular fluorescence complementation (BiFC) assay in leaf epidermal cells of *N. benthamiana*. Similar to OsRE1, OsRIP1 displayed a nucleus-localized pattern in rice protoplasts (Figure S3a). When OsRE1-eYNE was coexpressed with eYCE-OsRIP1, strong eYFP fluorescence signals were observed in the DAPI (4′, 6-diamino-2-phenylindole)-labelled nucleus (Figure 4c). In addition, a firefly luciferase complementation imaging (LCI) assay confirmed the interaction in leaves of *N. benthamiana* (Figure S2c). Furthermore, an *in vivo* co-immunoprecipitation (Co-IP) assay showed that Flag-OsRIP1 could be co-immunoprecipitated by OsRE1-GFP but not free GFP in total leaf extract of *N. benthamiana* (Figure 4d).

qRT-PCR analyses showed that *OsRIP1* displayed a similar diurnal expression pattern with *OsRE1* (Figure S3b-d). To investigate whether *OsRIP1* is authentically involved in heading, we generated the *Osrip1* mutant using the CRISPR-*Cas9* technology. Two homozygous *Osrip1* mutant lines with different editing (−1 bp and +1 bp) were selected for further study (Figure 5a).

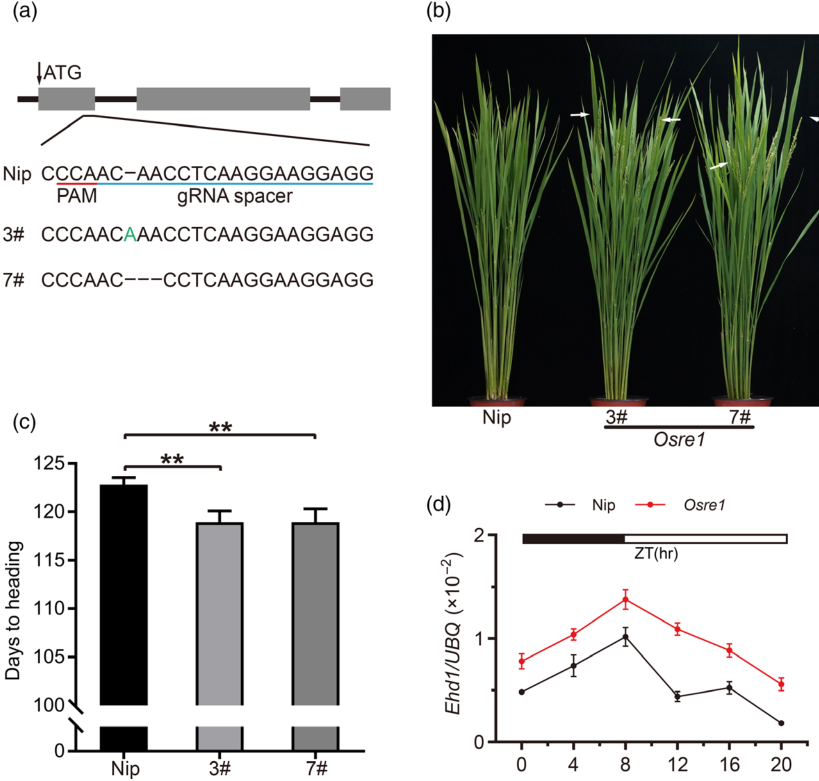

Figure 2 Loss of *OsRE1* function promotes flowering. (a) Mutation sites in *OsRE1* modified by the CRISPR-*Cas9* genome editing. The guide RNA (gRNA) spacer sequence is shown in blue, and the protospacer adjacent motif (PAM) site is shown in red. Deleted nucleotides are depicted as dashes, and inserted nucleotides are shown in green. Nip, Nipponbare. (b) Phenotypes of Nip and *Osre1* under NLD conditions. 3# and 7# represent two independent *Osre1* mutant lines. White arrows indicate panicles. Nip, Nipponbare. (c) Statistical analysis for heading date of Nip and *Osre1*; the mutants showing earlier flowering. Values are means ± SD; $n > 20$. Asterisks indicate significantly different values (**, $P < 0.01$). Nip, Nipponbare. (d) Rhythmic expression patterns of *Ehd1* in Nip and *Osre1* under LD conditions. The plants were grown in growth chambers under LD (14-h light/10-h darkness) conditions for 50 days. Black and white boxes denote dark and light periods, respectively. Rice *UBIQUITIN* gene was used as the internal control. Values are means ± SD; $n = 3$. ZT, zeitgeber time. Nip, Nipponbare. [Colour figure can be viewed at wileyonlinelibrary.com]

These lines flowered 8.5 days earlier than Nipponbare when grown under NLD conditions in Beijing (Figure 5b, c). Agronomic traits including plant height, panicle length and grains per panicle were reduced relative to Nipponbare, but 1000-grain weight is not unaffected (Table S1). Together, these results suggested that *OsRIP1* is required for flowering in rice.

OsRIP1 works cooperatively with OsRE1 to repress the transcript expression of *Ehd1*

The expression level of *Ehd1* was also higher in the *Osrip1* mutant than in WT, suggesting that OsRIP1 participates in the *Ehd1* pathway (Figure 5d). To determine whether OsRIP1 directly binds to the *Ehd1* promoter, we performed Y1H and the result showed that OsRIP1 could not bind the *Ehd1* promoter (Figure S4). An *in vitro* EMSA and *in vivo* ChIP assay further confirmed OsRIP1 could not bind the *Ehd1* promoter (Figure 6a, b). Given that the physical interaction between OsRIP1 and OsRE1, we proposed that OsRIP1 may function cooperatively with OsRE1 to repress the transcriptional activity of *Ehd1*. A quantitative transactivation assay was performed to investigate the regulatory relationship of OsRE1 and OsRIP1 in modulating the activity of *Ehd1*. A 2000 bp fragment upstream from start codon of *Ehd1* was fused to LUC as the reporter. OsRE1-GFP and OsRIP1-Flag were used as effectors with GFP and 3 × Flag as controls (Figure 6c). Luciferase (LUC) activity was reduced by one-half when Pro_{Ehd1}:LUC was co-transfected with 35S-OsRE1-GFP. Strikingly, when both OsRE1 and OsRIP1 proteins were coexpressed, the LUC activity declined to one-third of the control (Figure 6d), suggesting that OsRIP1 facilitates OsRE1 to repress the transcript expression of *Ehd1*.

Genetic interaction between *OsRE1*, *OsRIP1* and *Ehd1*

To further investigate the genetic interactions between *OsRE1, OsRIP1* and *Ehd1*, we generated *Osre1 ehd1* and *Osrip1 ehd1* double mutants. Homozygous *Osre1 ehd1* and *Osrip1 ehd1* double mutants were selected by PCR-based genotyping (Figure S5). As shown in Figure 7, both *Osre1 ehd1* and *Osrip1 ehd1* double mutants show a more similar phenotype with *ehd1* than with Nipponbare and each single mutant, suggesting that *OsRE1* and *OsRIP1* function upstream of *Ehd1*. Additionally, we also noted the subtle difference between double mutants and *ehd1* (Figure 7), suggesting the possible existence of unknown targeted genes of OsRE1 and OsRIP1 in regulating heading date independent of *Ehd1*. To further investigate the downstream genes regulated by *OsRE1* and *OsRIP1*, qRT-PCR assays were performed. We found the expression of *Hd3a*, *RFT1*, *MADS14*

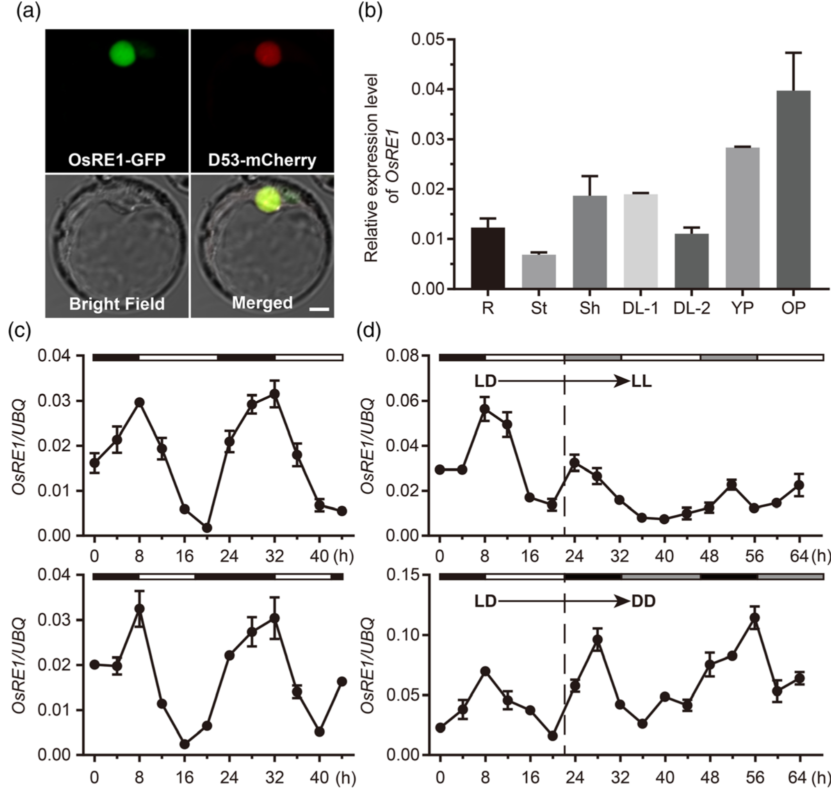

Figure 3 Subcellular localization of OsRE1 and expression profiles of *OsRE1*. (a) Subcellular localization of OsRE1 in rice protoplasts. D53-mCherry protein was used as a nuclear marker. Scale bar, 5 μm. (b) qRT-PCR analysis of *OsRE1* expression levels in different tissues. R, root; St, stem; Sh, sheath; DL-1, uppermost leaves; DL-2, second uppermost leaves; YP, young panicles; OP, old panicles. Values are means ± SD; n = 3. (c) Rhythmic expression of *OsRE1* in second uppermost leaves under LD (14-h light/10-h darkness) and SD (10-h light/14-h darkness) conditions. Rice *UBIQUITIN* gene was used as the internal control. Values are means ± SD; n = 3. (d) Expression analysis of *OsRE1* in second uppermost leaves under continuous light (LL) and continuous darkness (DD) conditions. The plants were grown in growth chambers under LD conditions for 30 days and then transferred to LL or DD conditions for 2 days. Black and white bars indicate darkness and light, respectively. Grey bars represent the subjective darkness in DD and subjective light in LL. Rice *UBIQUITIN* gene was used as the internal control. Values are means ± SD; n = 3. [Colour figure can be viewed at wileyonlinelibrary.com]

and *Ghd7* was also up-regulated in the *Osre1* and *Osrip1* mutant (Figure S6).

Discussion

Although previous studies showed many heading date genes were cloned, most of them are major genes, hard to be utilized in rice breeding. Minor-effect genes regulate rice heading date weakly, which can regulate rice flowering at the right time, and let rice make full use of the conditions of light and temperature, so these genes can be used for rice heading date breed improvement. Here, we identified OsRE1 as a direct regulator of *Ehd1*. OsRE1 was annotated as OsbZIP1 in past study, inducible in response to infection with *Magnaporthe grisea* (Meng *et al*., 2005). In our study, we found OsRE1 participated in the rice heading date pathway. Furthermore, we found that OsRIP1, a BBX-like transcription factor, interacts with OsRE1 *in vitro* and *in vivo*. Both proteins were located in the nucleus and exhibited similar rhythmical expressions. Loss-of-function mutations of *OsRE1* and *OsRIP1* promote heading date in rice. Our genetic data suggested that *OsRE1* and *OsRIP1* may function upstream of *Ehd1* in regulating heading date. Together, our combined data suggested that OsRE1 and OsRIP1 act as two minor-effect negative regulators of heading date in rice.

OsRE1 represses *Ehd1* expression by directly binding to its promoter

Ehd1 is the key gene in a unique heading date pathway in rice. Although many genes participate in the *Ehd1* pathway, direct regulators of *Ehd1* largely remain to be identified. Previous studies showed that OsLFL1, a putative B3 transcription factor, binds to RY *cis*-elements (CATGCATG) in the *Ehd1* promoter (Peng *et al*., 2007). *OsLFL1* functions downstream of *OsMADS50* and *OsMADS56* to repress the expression of *Ehd1* (Ryu *et al*., 2009). In this study, our EMSA and ChIP assay showed that OsRE1 can directly bind to the *Ehd1* promoter (Figure 1). Furthermore, an A-box motif 638 bp upstream of the ATG of *Ehd1* was determined to be the binding site of OsRE1 (Figure 1). The binding site of OsLFL1 (−883 bp and −557 bp) is close to that of OsRE1 (−638 bp). The ACGT motif is also a potential binding site for B3 proteins (Suzuki *et al*., 1997). Both OsLFL1 and OsRE1 can repress the expression of *Ehd1*. It will be an interesting avenue to determine whether OsRE1 functions cooperatively with or independently of OsLFL1 in repressing the *Ehd1* expression. The C2H2 zinc finger protein SIP1 is another direct regulator of *Ehd1* (Jiang *et al*., 2018). SIP1 participates in regulation of heading date in rice by recruiting OsTrx1 to *Ehd1*. We found that OsRE1 cannot interact with SIP1 in yeast (data not shown), suggesting that

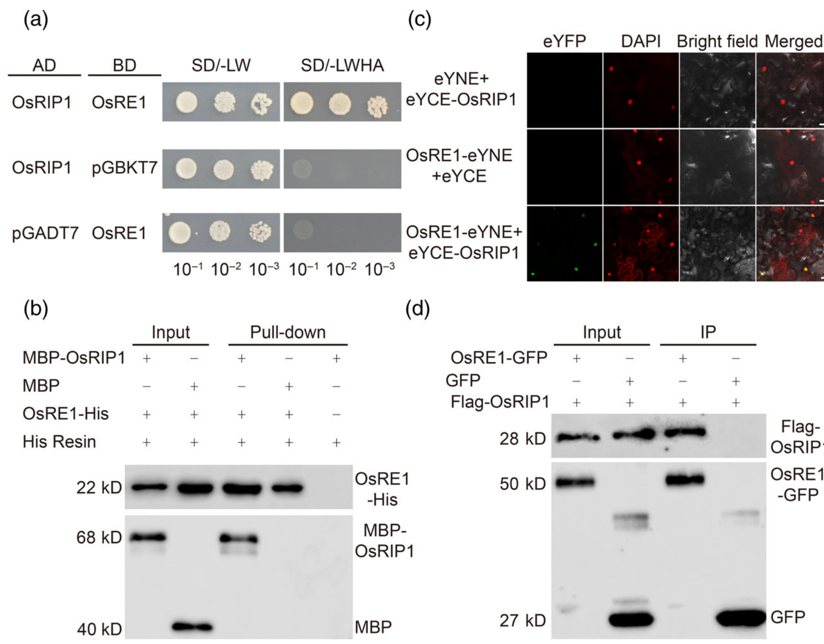

Figure 4 OsRE1 physically interacts with OsRIP1. (a) Yeast two-hybrid assay showed that OsRE1 interacts with OsRIP1. Yeast was grown at 30 °C for 3 days. Empty vectors were used as the negative controls. AD, activation domain. BD, binding domain. (b) *In vitro* pull-down assay showed that OsRE1-His pulls down MBP-OsRIP1 but not free MBP. Free MBP and His resin were used as the negative controls. (c) Bimolecular fluorescence complementation (BiFC) assays showed OsRE1 interacts with OsRIP1 in nucleus (labelled by fluorescence of 4′, 6-diamino-2-phenylindole) in leaf epidermal cells of *N. benthamiana*. Scale bars, 20 μm. (d) *In vivo* Co-IP assay showed that Flag-OsRIP1 can be co-immunoprecipitated in the total leaf extract *N. benthamiana* expressing OsRE1-GFP but not free GFP using anti-GFP agarose beads. [Colour figure can be viewed at wileyonlinelibrary.com]

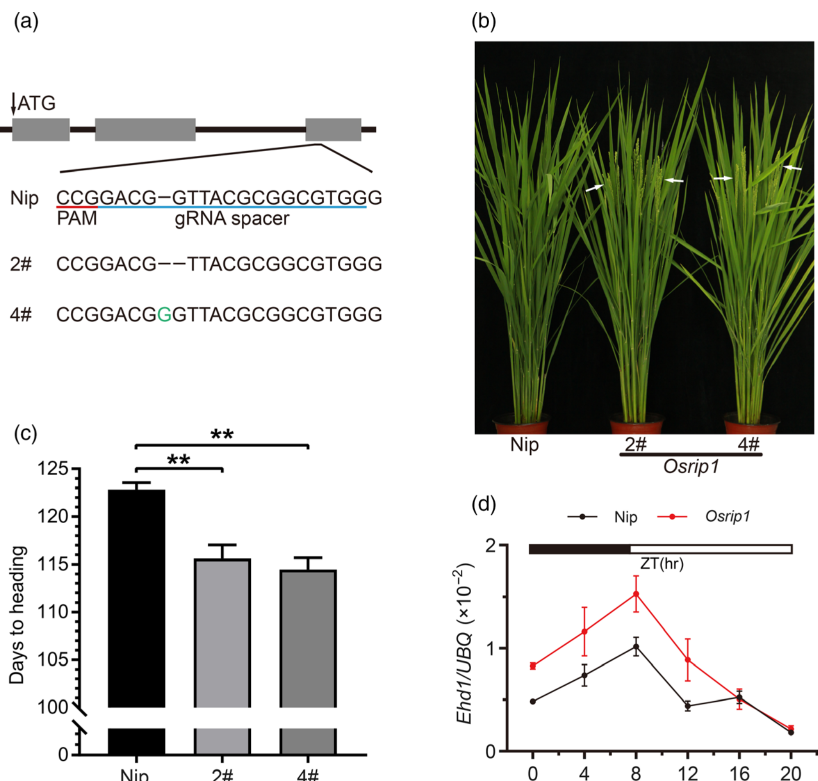

Figure 5 Loss-of-function mutation of *OsRIP1* promotes flowering. (a) Mutation sites in *OsRIP1* induced by the CRISPR-*Cas9* genome editing system. The guide RNA (gRNA) spacer sequence is shown in blue, and the protospacer adjacent motif (PAM) site is shown in red. Deleted nucleotides are depicted as dashes, and inserted nucleotides are shown in green. (b) Phenotypes of Nip and *Osrip1* mutant under NLD conditions; 2# and 4# represent two independent *Osrip1* mutant lines. White arrows indicate panicles. Nip, Nipponbare. (c) Statistical data for heading date of Nip and *Osrip1* mutant under NLD conditions. Nip, Nipponbare. Values are means ± SD; $n > 20$. Asterisks indicate significantly different values (**, $P < 0.01$). (d) Rhythmic expression patterns of *Ehd1* in Nip and *Osrip1* backgrounds under LD conditions. The plants were grown in growth chambers under LD (14-h light/10-h darkness) conditions for 50 days. Black and white boxes denote dark and light periods, respectively. Rice *UBIQUITIN* gene was used as the internal control. Values are means ± SD; $n = 3$. ZT, Zeitgeber time. Nip, Nipponbare. [Colour figure can be viewed at wileyonlinelibrary.com]

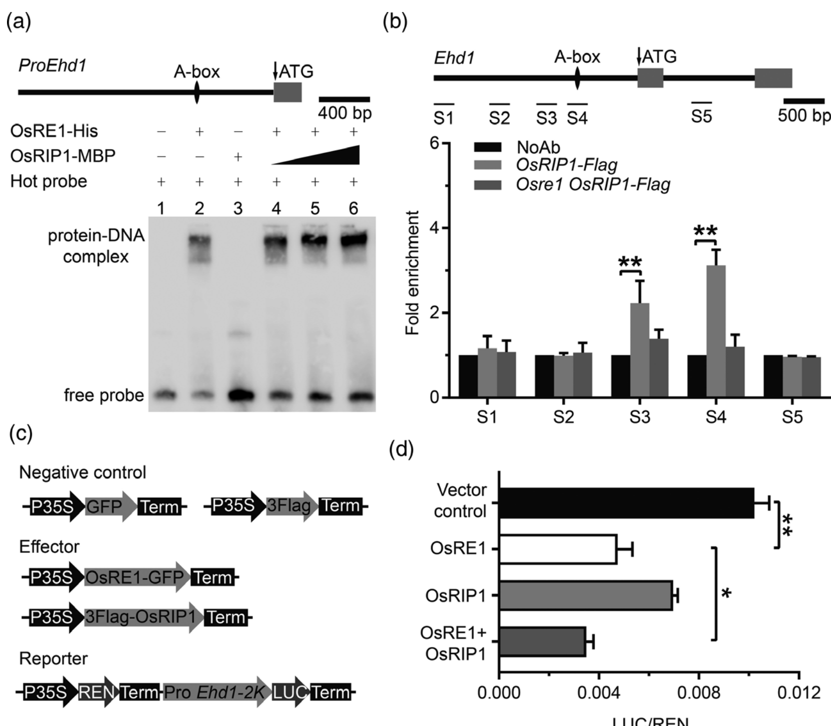

Figure 6 OsRE1 functions cooperatively with OsRIP1 to negatively regulate *Ehd1* expression. (a) EMSA showed that OsRIP1 cannot directly bind to but facilitate OsRE1 to bind to the A-box motif of the *Ehd1* promoter. The hot probe was a biotin-labelled A-box motif-containing fragment in the *Ehd1* promoter (sequence showed in Figure 1c). (b) ChIP-qPCR assay showed *in vivo* binding of OsRIP1 to the *Ehd1* promoter is dependent on OsRE1. Cross-linked chromatin samples were extracted from *Flag-OsRIP1* and *osre1/Flag-OsRIP1* transgenic plants and then precipitated with Flag antibody. NoAb (No antibody) served as a negative control. Values are means ± SD; $n = 3$. (c) Schematic diagram of various constructs used in the transient transformation assay. 35S: REN-ProEhd1: LUC was constructed as the reporter. 35S: OsRE1-GFP and 35S:3 × Flag-OsRIP1 were constructed as effectors. Free GFP and Flag were used as negative controls. (d) *In vivo* luciferase assay verified the inhibitory effect of OsRE1 and OsRIP1 on *Ehd1* expression in *N. benthamiana* leaves. Expression level of Renilla (REN) was used as an internal control. The LUC/REN ratio represents the relative activity of the Ehd1 promoter. Values are means ± SD; $n = 3$. Asterisks indicate significantly different values (*, $P < 0.05$; **, $P < 0.01$).

OsRE1 may not be involved in the pathway of SIP1-mediated *Ehd1* regulation.

Recent studies showed that Hd1 also represses expression of *Ehd1* through interaction with Ghd7 (Nemoto *et al*., 2016), but whether Hd1 and Ghd7 could bind to the *Ehd1* promoter directly was not clear. DTH8 interacts with Hd1 to repress *Hd3a* expression by changing the H3K27me3 level in the *Hd3a* promoter (Du *et al*., 2017). Ghd7 can also interact with DTH8. It was reported that Ghd7, Hd1 and DTH8 can form a protein complex (Cai *et al*., 2019). Whether the Ghd7-Hd1-DTH8 complex interacts with the *Ehd1* promoter is yet to be established.

DTH8 was reported to down-regulate expression of *Ehd1* indirectly (Wei *et al*., 2010), but the detailed mechanism was not clear. In this study, OsRE1 directly interacts with DTH8 but not with Ghd7 or Hd1 (Figure S7a, b), and OsRE1 represses expression of *Ehd1* (Figure 2d). The expression level of *OsRE1* was upgraded in the *dth8* mutant, while it was not changed in *ghd7* mutant (Figure S7c). Therefore, OsRE1 might function as a bridge between DTH8 and *Ehd1* in regulating heading date in rice, as the reported DTH8-Hd1 module (Du *et al*., 2017; Zhu *et al*., 2017).

OsRIP1 delays heading date in an OsRE1-dependent manner

OsRIP1 was previously annotated as OsBBX13 (Huang *et al*., 2012), but the exact function remains unknown. BBX proteins have been reported to play important roles in plant growth and development, including photoperiodic regulation of flowering (Cheng and Wang, 2005; Crocco *et al*., 2015; Gangappa *et al*., 2013; Kumagai *et al*., 2008). Here, we found that OsRIP1 regulates rice heading date by repressing *Ehd1* expression in an OsRE1-dependent manner. Both *Osre1* and *Osrip1* mutants showed the early heading phenotype than wild-type Nipponbare (Figures 2; 5). The *Osre1 ehd1* and *Osrip1 ehd1* double mutants flowered much later than Nipponbare, *Osre1* and *Osrip1* (Figure 7). These findings suggested that OsRE1 and OsRIP1 genetically function upstream of *Ehd1*. Consistently, genes downstream of *Ehd1*, such as *Hd3a*, *RFT1* and *OsMADS14*, were promoted in the loss-of-function mutants while genes regulating *Ehd1*, such as *DTH8* and *Ehd3*, were unaffected obviously in the transgenic plants (Figure S6).

Together, our study identified *OsRE1* and *OsRIP1* as two minor-effect genes in regulating heading date and thus provides the putative application in crop adaptation and genetic breeding.

Materials and Methods

Plant materials and growth conditions

Transgenic plants were produced using the *Oryza sativa* subsp. *japonica* varieties, Nipponbare. All plants were grown in the experimental field at Beijing (40°13′N, 116°34′E) under natural long-day (NLD) conditions. Plants were also grown in light incubators with constant 70% relative humidity and about

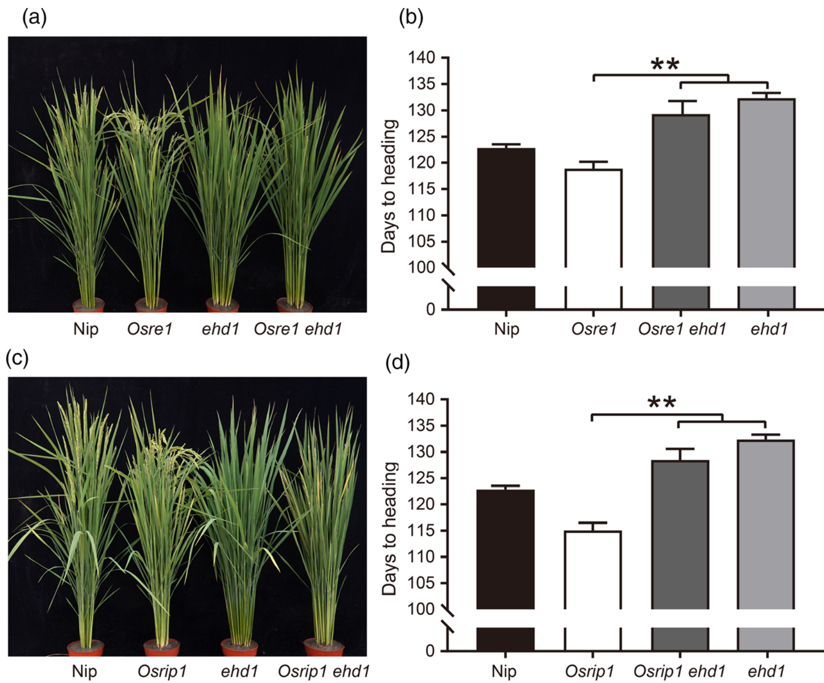

Figure 7 *Osre1 ehd1* and *Osrip1 ehd1* double mutants show a more similar phenotype with *ehd1*. (a) Phenotypes of Nip, *Osre1*, *ehd1* and *Osre1 ehd1* at heading stage under NLD conditions. Nip, Nipponbare. (b) Statistical analyses of days to heading of Nip, *Osre1*, *ehd1* and *Osre1 ehd1* plants under NLD conditions. Nip, Nipponbare. Values are means ± SD; $n > 20$. NLD, natural long days. (c) Phenotypes of Nip, *Osrip1*, *ehd1* and *Osrip1 ehd1* at the heading stage under NLD conditions. Nip, Nipponbare. (d) Statistical analyses of days to heading of Nip, *Osrip1*, *ehd1* and *Osrip1 ehd1* under NLD conditions. Nip, Nipponbare. Values are means ± SD; $n > 20$. NLD, natural long days. [Colour figure can be viewed at wileyonlinelibrary.com]

800 μmol/m^2/s light intensity under artificial LD (14-h light, 30°C/10-h dark, 25°C) or SD (10-h light, 30°C/14-h dark, 25°C) conditions.

Generation of transgenic plants

To generate *Osre1* and *Osrip1* mutants, 18 bp gene-specific spacer sequences for target genes (Table S2) were inserted into the entry vector pOs-sgRNA, respectively, followed by subcloning into the destination vector of Cas9 by using the Gateway LR Clonase II Enzyme mix (Invitrogen; Miao *et al*., 2013). Transgenic plants were PCR-genotyped, and insertion/deletion mutants at the target site were isolated by DNA sequencing. The primer sequences for construction of these vectors are provided in Table S2.

Yeast one-hybrid assay

For yeast one-hybrid assay, the coding sequence (CDS) of *OsRE1* was amplified by PCR and cloned into the pB42AD vector (Clontech). To generate Pro_{Ehd1}*: LacZ* reporter gene, several fragments of *Ehd1* promoter were amplified and cloned into the vector pLacZi. Plasmids were co-transformed into yeast strain EGY48. Transformants were selected and grown on SD/-Trp-Ura dropout medium for 72 h, and then transferred onto X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) plates for blue colour development (Lin *et al*., 2007).

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP assay was performed as previously described (Zhang *et al*., 2012). Uppermost leaves of *Pro35S:3* × *Flag-OsRE1* transgenic plants were used for ChIP assay. Flag antibodies (Medical Biological Laboratories, PM020) were used for detection. The precipitated DNA was analysed by RT-qPCR using primers listed in Table S2.

Electrophoretic mobility shift assay (EMSA)

The LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) was used for EMSA. Oligonucleotide probes were synthesized and labelled with biotin by Thermo Fisher Scientific. The OsRE1-His and MBP-OsRIP1 fusion proteins were expressed in *Escherichia coli* strain BL21. The recombinant proteins were purified using either IDA-Nickel Beads (BEAVER) or amylose resin (BioLabs) affinity chromatography. EMSA was performed according to the manufacturer's instructions (Thermo, No. 20148). Primer sequences used in EMSA are provided in Table S2.

Subcellular localization

The CDSs of *OsRE1* and *OsRIP1* were fused at the N-terminal with GFP in the pAN580 vector, respectively. The GFP fusion plasmids were transformed into rice protoplasts as described previously (Zhang *et al*., 2011). *Pro35S:D53-mCherry* was used as a nucleus marker (Zhou *et al*., 2013). Fluorescence signals were detected by a ZESS LSM880 confocal microscope.

Yeast two-hybrid assay

For the yeast two-hybrid assay, CDS and truncated fragments of *OsRE1* and *OsRIP1* were amplified and cloned into the pGADT7 or pGBKT7 plasmids (Clontech), respectively. The empty pGADT7 or pGBKT7 vectors were used as negative controls. Yeast co-transformation was performed following the manual of the Matchmaker Gold Yeast Two-Hybrid System (Clontech). Different yeast strains were grown on DDO (SD/-Leu-Trp) plates and QDO

(SD/-Leu-Trp-Ade-His) plates. The interactions were observed after 3 d of incubation at 30 °C.

Bimolecular fluorescence complementation (BiFC) assays

The CDSs of *OsRE1* and *OsRIP1* were fused to YNE or YCE of pDOE-02 vectors, respectively (Gookin and Assmann, 2014). The plasmids were transformed into *Agrobacterium tumefaciens* (strain EHA105) and infiltrated into *Nicotiana benthamiana* leaves as described previously (Waadt and Kudla, 2008). Fluorescence signals were detected by a ZEISS LSM880 confocal microscope.

Pull-down assays

For the pull-down assay, the CDS of *OsRE1* was fused to the prokaryotic expression vector pET-30a to express OsRE1-His. The CDS of *OsRIP1* was inserted into pMAL-c2X to express MBP-OsRIP1. Plasmids were transformed into *E. coli* BL21. Fusion proteins were induced with 0.5 mM IPTG at 16 °C for 16 h. Pull-down assays were performed as reported previously (Miernyk and Thelen, 2008). Anti-His (Medical Biological Laboratories, 1:2000) and anti-MBP (New England Biolabs, 1:2000) antibodies were used in immunoblotting analysis.

Co-immunoprecipitation (Co-IP) assay

The CDS of *OsRE1* was fused to pCAMBIA1305.1-GFP vector. The CDS of *OsRIP1* was inserted into pCAMBIA1300-221-Flag vector. The plasmids were co-transformed into *Agrobacterium tumefaciens* (strain EHA105) and then infiltrated into *N. benthamiana* leaves. Anti-GFP and anti-Flag antibodies (Medical Biological Laboratories, 1:2000) were used in immunoblotting analysis.

Luciferase complementation image (LCI) assay

The CDSs of *OsRE1* and *OsRIP1* were fused to the pCAMBIA1300-nLUC and pCAMBIA1300-cLUC vectors, respectively, for the LCI assay. The empty vectors were used as negative controls. The plasmids were co-transformed into *Agrobacterium tumefaciens* (strain EHA105) and then infiltrated into *N. benthamiana* leaves. CCD imaging apparatus (Berthold, LB985) was used to capture the LUC image. The injected leaves were sprayed with 10 mM beetle luciferin (Promega, E1602) and placed in darkness for 5 min before luminescence detection.

Quantitative transactivation assay

For the quantitative transactivation assay, the 2000 bp promoter of *Ehd1* was cloned to fuse into the vector pGreenII0800-LUC as the reporter. The OsRE1-GFP and Flag-OsRIP1 fusion proteins were used as the effectors. Empty vectors were used as negative controls. The above plasmids were co-transformed into *Agrobacterium tumefaciens* (strain EHA105) and then infiltrated into *N. benthamiana* leaves. The Dual-Glo Luciferase Assay System (E2920, Promega) was used to measure the luciferase (LUC) activity.

Phylogenetic analysis

Gene sequences used in phylogenetic analysis were downloaded and aligned using Geneious (v4.8.5). Alignment data were used to perform maximum-likelihood analysis. Bootstrap values were estimated from bootstrap analyses of 1000 replicates.

Real-time PCR assays

Total RNAs of organs were extracted using an RNAprep Pure Kit (Zymo Research, Orange, CA) and were reversely transcribed using a Reverse Transcription Kit (Qiagen). qRT-PCR was performed using a SYBR Premix Ex Taq II Kit (TaKaRa) in an ABI PRISM 7900HT. Rice *UBIQUITIN* was used as internal control. Data from three biological replicates were analysed as described previously (Livak and Schmittgen, 2001).

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Wan Jianmin and Zhu Shanshan designed the research. Chai Juntao, Zhu Shanshan and Li Chaonan performed most of the experiments. Zheng Xiaoming, Zhou Shirong, Cai Maohong and Zhou Liang helped to analysed data. Zhang Huan, Sheng Peike and Wu Mingming helped to generate the double mutants. Cheng Zhijun, Lei Cailin, Wang Jie, Zhao Zhichao and Jin Xin conducted fieldwork. Wang Chunming, Ren Yulong, Lin Qibing, Guo Xiuping and Zhang Xin provided technical support. Chai Juntao, Zhu Shanshan and Li Chaonan contributed equally.

References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H. *et al*. (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science*, **309**, 1052.

Bai, B., Zhao, J., Li, Y., Zhang, F., Zhou, J., Chen, F. and Xie, X. (2016) *OsBBX14* delays heading date by repressing florigen gene expression under long and short-day conditions in rice. *Plant Sci.* **247**, 25–34.

Borden, K.L.B. (1998) RING fingers and B-boxes: zinc-binding protein-protein interaction domains. *Biochem. Cell Biol.* **76**, 351–358.

Brambilla, V., Martignago, D., Goretti, D., Cerise, M., Somssich, M., de Rosa, M., Galbiati, F. *et al*. (2017) Antagonistic transcription factor complexes modulate the floral transition in rice. *Plant Cell*, **29**, 2801.

Cai, M., Chen, S., Wu, M., Zheng, T., Zhou, L., Li, C., Zhang, H. *et al*. (2019) Early heading 7 interacts with DTH8, and regulates flowering time in rice. *Plant Cell Rep.* **38**, 521–532.

Cheng, X.-F. and Wang, Z.-Y. (2005) Overexpression of *COL9*, a *CONSTANS-LIKE* gene, delays flowering by reducing expression of *CO* and *FT* in *Arabidopsis thaliana*. *Plant J.* **43**, 758–768.

Crocco, C.D., Locascio, A., Escudero, C.M., Alabadí, D., Blázquez, M.A. and Botto, J.F. (2015) The transcriptional regulator BBX24 impairs DELLA activity to promote shade avoidance in *Arabidopsis thaliana*. *Nat. Commun.* **6**, 6202.

Doi, K., Izawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., Yano, M. *et al*. (2004) *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1*. *Genes Develop.* **18**, 926–936.

Dröge-Laser, W., Snoek, B.L., Snel, B. and Weiste, C. (2018) The *Arabidopsis* bZIP transcription factor family—an update. *Curr. Opin. Plant Biol.* **45**, 36–49.

Du, A., Tian, W., Wei, M., Yan, W., He, H., Zhou, D., Huang, X. *et al*. (2017) The DTH8-Hd1 module mediates day-length-dependent regulation of rice flowering. *Mol. Plant,* **10**, 948–961.

E, Z.G., Zhang, Y.P., Zhou, J.H. and Wang, L. (2014) Mini review roles of the *bZIP* gene family in rice. *Genetics Mol. Res.* **13**, 3025–3036.

Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M., Mueller, N.D. *et al*. (2011) Solutions for a cultivated planet. *Nature*, **478**, 337.

Gangappa, S.N. and Botto, J.F. (2014) The BBX family of plant transcription factors. *Trends Plant Sci.* **19**, 460–470.

Gangappa, S.N., Crocco, C.D., Johansson, H., Datta, S., Hettiarachchi, C., Holm, M. and Botto, J.F. (2013) The *Arabidopsis* B-BOX protein BBX25 interacts with HY5, negatively regulating *BBX22* expression to suppress seedling photomorphogenesis. *Plant Cell*, **25**, 1243–1257.

Gao, H., Zheng, X.-M., Fei, G., Chen, J., Jin, M., Ren, Y., Wu, W. *et al*. (2013) *Ehd4* encodes a novel and *Oryza*-genus-specific regulator of photoperiodic flowering in rice. *PLOS Genet.* **9**, e1003281.

Gookin, T.E. and Assmann, S.M. (2014) Significant reduction of BiFC non-specific assembly facilitates in planta assessment of heterotrimeric G-protein interactors. *Plant J.* **80**, 553–567.

Griffiths, S., Dunford, R.P., Coupland, G. and Laurie, D.A. (2003) The evolution of *CONSTANS-like* gene families in barley, rice, and *Arabidopsis*. *Plant Physiol.* **131**, 1855–1867.

Hartmann, U., Sagasser, M., Mehrtens, F., Stracke, R. and Weisshaar, B. (2005) Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. *Plant Mol. Biol.* **57**, 155–171.

Hayama, R., Izawa, T. and Shimamoto, K. (2002) Isolation of rice genes possibly involved in the photoperiodic control of flowering by a fluorescent differential display method. *Plant Cell Physiol.* **43**, 494–504.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature* **422**, 719–722.

Huang, J., Zhao, X., Weng, X., Wang, L. and Xie, W. (2012) The rice B-box zinc finger gene family: genomic identification, characterization, expression profiling and diurnal analysis. *PLoS One* **7**, e48242.

Izawa, T. (2007) Adaptation of flowering-time by natural and artificial selection in *Arabidopsis* and rice. *J. Exp. Botany*, **58**, 3091–3097.

Izawa, T., Foster, R. and Chua, N.H. (1994) Plant bZIP proteins gather at ACGT elements. *FASEB J.* **8**, 192–200.

Jiang, P., Wang, S., Zheng, H., Li, H., Zhang, F., Su, Y., Xu, Z. *et al*. (2018) SIP1 participates in regulation of flowering time in rice by recruiting OsTrx1 to *Ehd1*. *New Phytol.* **219**, 422–435.

Kim, S.L., Lee, S., Kim, H.J., Nam, H.G. and An, G. (2007) *OsMADS51* Is a short-day flowering promoter that functions upstream of *Ehd1, OsMADS14* and *Hd3a*. *Plant Physiol.* **145**, 1484.

Kim, S.-K., Yun, C.-H., Lee, J.H., Jang, Y.H., Park, H.-Y. and Kim, J.-K. (2008) *OsCO3*, a *CONSTANS-LIKE* gene, controls flowering by negatively regulating the expression of *FT*-like genes under SD conditions in rice. *Planta* **228**, 355.

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T. and Yano, M. (2002) *Hd3a*, a rice ortholog of the *Arabidopsis FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant Cell Physiol.* **43**, 1096–1105.

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S. and Shimamoto, K. (2008) *Hd3a* and *RFT1* are essential for flowering in rice. *Development*, **135**, 767.

Kumagai, T., Ito, S., Nakamichi, N., Niwa, Y., Murakami, M., Yamashino, T. and Mizuno, T. (2008) The common function of a novel subfamily of B-Box zinc finger proteins with reference to circadian-associated events in *Arabidopsis thaliana*. *Biosci., Biotechnol. Biochem.* **72**, 1539–1549.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science*, **240**, 1759.

Lee, S., Kim, J., Han, J.-J., Han, M.-J. and An, G. (2004) Functional analyses of the flowering time gene *OsMADS50*, the putative *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20)* ortholog in rice. *Plant J.* **38**, 754–764.

Lee, Y.-S., Jeong, D.-H., Lee, D.-Y., Yi, J., Ryu, C.-H., Kim, S.L., Jeong, H.J. *et al*. (2010) *OsCOL4* is a constitutive flowering repressor upstream of *Ehd1* and downstream of *OsphyB*. *Plant J.* **63**, 18–30.

Lee, Y.-S., Lee, D.-Y., Cho, L.-H. and An, G. (2014) Rice miR172 induces flowering by suppressing *OsIDS1* and *SNB*, two AP2 genes that negatively regulate expression of *Ehd1* and florigens. *Rice*, **7**, 31.

Lin, R., Ding, L., Casola, C., Ripoll, D.R., Feschotte, C. and Wang, H. (2007) Transposase-derived transcription factors regulate light signaling in *Arabidopsis*. *Science*, **318**, 1302–1305.

Liu, H., Gu, F., Dong, S., Liu, W., Wang, H., Chen, Z. and Wang, J. (2016) CONSTANS-like 9 (COL9) delays the flowering time in Oryza sativa by repressing the *Ehd1* pathway. *Biochem. Biophys. Res. Commun.* **479**, 173–178.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta$CT method. *Methods*, **25**, 402–408.

Luan, W., Chen, H., Fu, Y., Si, H., Peng, W., Song, S., Liu, W. *et al*. (2009) The effect of the crosstalk between photoperiod and temperature on the heading-date in rice. *PLoS One*, **4**, e5891.

Matsubara, K., Yamanouchi, U., Wang, Z.-X., Minobe, Y., Izawa, T. and Yano, M..(2008) *Ehd2* a rice ortholog of the maize *INDETERMINATE1* gene, promotes flowering by up-regulating *Ehd1*. *Plant Physiol.* **148**, 1425.

Matsubara, K., Yamanouchi, U., Nonoue, Y., Sugimoto, K., Wang, Z.-X., Minobe, Y. and Yano, M. (2011) *Ehd3*, encoding a plant homeodomain finger-containing protein, is a critical promoter of rice flowering. *Plant J.* **66**, 603–612.

Meng, X.B., Zhao, W.S., Lin, R.M. and Wang, M. (2005) Identification of a novel rice bZIP-type transcription factor gene, OsbZIP1, involved in response to infection of *Magnaporthe grisea*. *Plant Mol. Biol. Reporter*, **23**, 301–302.

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., Wan, J. *et al*. (2013) Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res.* **23**, 1233–1236.

Miernyk, J.A. and Thelen, J.J. (2008) Biochemical approaches for discovering protein–protein interactions. *Plant J.* **53**, 597–609.

Nemoto, Y., Nonoue, Y., Yano, M. and Izawa, T. (2016) Hd1, a CONSTANS ortholog in rice, functions as an *Ehd*1 repressor through interaction with monocot-specific CCT-domain protein Ghd7. *Plant J.* **86**, 221–233.

Nijhawan, A., Jain, M., Tyagi, A.K. and Khurana, J.P. (2008) Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice. *Plant Physiol.* **146**, 333.

Peng, L.-T., Shi, Z.-Y., Li, L., Shen, G.-Z. and Zhang, J.-L. (2007) Ectopic expression of OsLFL1 in rice represses *Ehd1* by binding on its promoter. *Biochem. Biophys. Res. Commun.* **360**, 251–256.

Ryu, C.-H., Lee, S., Cho, L.-H., Kim, S.L., Lee, Y.-S., Choi, S.C., Jeong, H.J. *et al*. (2009) *OsMADS50* and *OsMADS56* function antagonistically in regulating long day (LD)-dependent flowering in rice. *Plant, Cell Environ.* **32**, 1412–1427.

Sheng, P., Wu, F., Tan, J., Zhang, H., Ma, W., Chen, L., Wang, J. *et al*. (2016) A *CONSTANS-like* transcriptional activator, *OsCOL13*, functions as a negative regulator of flowering downstream of *OsphyB* and upstream of *Ehd1* in rice. *Plant Mol. Biol.* **92**, 209–222.

Shrestha, R., Gómez-Ariza, J., Brambilla, V. and Fornara, F. (2014) Molecular control of seasonal flowering in rice, *Arabidopsis* and temperate cereals. *Annals Botany*, **114**, 1445–1458.

Song, Y.H., Shim, J.S., Kinmonth-Schultz, H.A. and Imaizumi, T. (2015) Photoperiodic flowering: time measurement mechanisms in leaves. *Annual Rev. Plant Biol.* **66**, 441–464.

Song, S., Wang, G., Hu, Y., Liu, H., Bai, X., Qin, R. and Xing, Y. (2018) OsMFT1 increases spikelets per panicle and delays heading date in rice by suppressing *Ehd1*, *FZP* and *SEPALLATA-like* genes. *J. Exp. Botany*, **69**, 4283–4293.

Sun, C., Chen, D., Fang, J., Wang, P., Deng, X. and Chu, C. (2014) Understanding the genetic and epigenetic architecture in complex network of rice flowering pathways. *Protein Cell*, **5**, 889–898.

Suzuki, M., Kao, C.Y., McCarty, D.R. *et al*. (1997) The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell*, **9**, 799–807.

Tamaki, S., Matsuo, S., Wong, H.L., Yokoi, S. and Shimamoto, K. (2007) Hd3a protein is a mobile flowering signal in rice. *Science*, **316**, 1033.

Tan, J., Jin, M., Wang, J., Wu, F., Sheng, P., Cheng, Z., Wang, J. *et al*. (2016) OsCOL10, a CONSTANS-Like gene, functions as a flowering time repressor downstream of Ghd7 in rice. *Plant Cell Physiol.* **57**, 798–812.

Torok, M. and Etkin, L.D. (2001) Two B or not two B? Overview of the rapidly expanding B-box family of proteins. *Differentiation,* **67**, 63–71.

Waadt, R. and Kudla, J. (2008) In planta visualization of protein interactions using bimolecular fluorescence complementation (BiFC). *Cold Spring Harbor Protocols*, **2008**, pdb.prot4995.

Wei, X., Xu, J., Guo, H., Jiang, L., Chen, S., Yu, C., Zhou, Z. *et al*. (2010) *DTH8* suppresses flowering in rice, influencing plant height and yield potential simultaneously. *Plant Physiol.* **153**, 1747.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U. and Weigel, D. (2005) Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* **309**, 1056.

Wu, C., You, C., Li, C., Long, T., Chen, G., Byrne, M.E. and Zhang, Q. (2008) *RID1* encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice. *Proc. Natl. Acad. Sci.* **105**, 12915.

Xue, W., Xing, Y., Weng, X., Zhao, Y., Tang, W., Wang, L., Zhou, H. *et al*. (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. *Nat. Genet.* **40**, 761.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T. *et al*. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell,* **12**, 2473.

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P. *et al*. (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods,* **7**, 30.

Zhang, L., Cheng, Z., Qin, R., Qiu, Y., Wang, J.-L., Cui, X., Gu, L. *et al*. (2012) Identification and characterization of an epi-allele of FIE1 reveals a regulatory linkage between two epigenetic marks in rice. *Plant Cell*, **24**, 4407–4421.

Zhang, C., Liu, J., Zhao, T., Gomez, A., Li, C., Yu, C., Li, H. *et al*. (2016) A drought-inducible transcription factor delays reproductive timing in rice. *Plant Physiol.* **171**, 334.

Zhang, Z., Hu, W., Shen, G., Liu, H., Hu, Y., Zhou, X., Liu, T. *et al*. (2017) Alternative functions of Hd1 in repressing or promoting heading are determined by Ghd7 status under long-day conditions. *Scientific Rep.* **7**, 5388.

Zhou, F., Lin, Q., Zhu, L., Ren, Y., Zhou, K., Shabek, N., Wu, F. *et al*. (2013) D14-SCF(D3)-dependent degradation of D53 regulates strigolactone signalling. *Nature* **504**, 406–410.

Zhu, S., Wang, J., Cai, M., Zhang, H., Wu, F., Xu, Y., Li, C. *et al*. (2017) The OsHAPL1-DTH8-Hd1 complex functions as the transcription regulator to repress heading date in rice. *J. Exp. Botany,* **68**, 553–568.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic analysis of BBX family proteins in rice.
Figure S2 OsRE1 physically interacts with OsRIP1.
Figure S3 Expression profiles and subcellular localization of OsRIP1.
Figure S4 OsRIP1 cannot bind to the promoter of *Ehd1*.in Y1H assay.
Figure S5 Sequencing results of mutant sites in transgenic plants.
Figure S6 Rhythmic expression patterns of genes related to heading date in Nipponbare, *Osre1* or *Osrip1* under LD conditions.
Figure S7 Interaction relationships between OsRE1, OsRIP1 and several known *Ehd1* repressors.
Table S1 Phenotypic data for Nipponbare, *Osre1* and *Osrip1* mutants.
Table S2 Primers used in this study.
Table S3 Detailed information for rice BBX family members.