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Original Article

The clock component OsLUX regulates rice heading through recruiting OsELF3-1 and OsELF4s to repress *Hd1* and *Ghd7*

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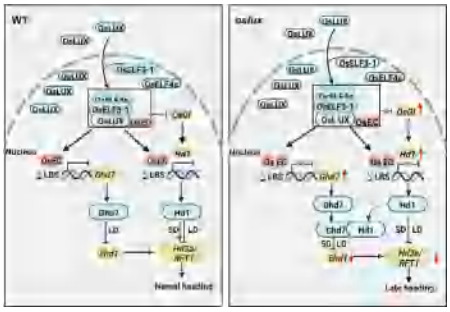
HIGHLIGHTS

- *OsLUX* regulates rice heading by repressing *Hd1* and *Ghd7*.
- Defect OsLUX causes extremely late heading phenotype dependent on *Hd1* and *Ghd7* under both photoperiod conditions.
- The cytoplasm-nuclear shuttling mechanism explains the effect of OsELF3-1 on OsLUX.
- The complete OsEC (OsELF4s–OsEL F3-1–OsLUX) complex is required to regulate heading via binding to the promoters of *Hd1* and *Ghd7*.
- The repressive strength of OsEC to regulate *Hd1* and *Ghd7* explained the different flowering effects of *OsEC* components.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Circadian clocks coordinate internal physiology and external environmental factors to regulate cereals flowering, which is critical for reproductive growth and optimal yield determination.
Objectives: In this study, we aimed to confirm the role of *OsLUX* in flowering time regulation in rice. Further research illustrates how the OsELF4s–OsELF3-1–OsLUX complex directly regulates flowering-related genes to mediate rice heading.
Methods: We identified a circadian gene *OsLUX* by the MutMap method. The transcription levels of flowering-related genes were evaluated in WT and *oslux* mutants. OsLUX forms OsEC (OsELF4s–OsEL F3-1–OsLUX) complex were supported by yeast two-hybrid, pull down, BiFC, and luciferase complementation assays (LCA). The EMSA, Chip-qPCR, luciferase luminescence images, and relative LUC activity

Abbreviations: Nip, Nipponbare; LDs, long-day conditions; SDs, short-day conditions; NLDs, natural long-day conditions; NSDs, natural short-day conditions; CLDs, controlled long-day conditions; CSDs, controlled short-day conditions; LL, continuous light; DD, continuous dark; ZT, zeitgeber time; CDS, coding sequence; Y2H, yeast two-hybrid; DDO, SD-Leu/-Trp; QDO, SD-Leu/-Trp/-His/-Ade; LCA, luciferase complementation assay; BiFC, bimolecular fluorescence complementation; EMSA, electrophoresis mobility shift assay; CCD, charge-coupled device.

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OsLUX
OsEC

assays were performed to examine the targeted regulation of flowering genes by the OsEC (OsELF4s–OsELF3-1–OsLUX) complex.

Results: The circadian gene *OsLUX* encodes an MYB family transcription factor that functions as a vital circadian clock regulator and controls rice heading. Defect in *OsLUX* causes an extremely late heading phenotype under natural long-day and short-day conditions, and the function was further confirmed through genetic complementation, overexpression, and CRISPR/Cas9 knockout. OsLUX forms the OsEC (OsELF4s–OsELF3-1–OsLUX) complex by recruiting OsELF3-1 and OsELF4s, which were required to regulate rice heading. OsELF3-1 contributes to the translocation of OsLUX to the nucleus, and a compromised flowering phenotype results upon mutation of any component of the OsEC complex. The OsEC complex directly represses *Hd1* and *Ghd7* expression via binding to their promoter's LBS (LUX binding site) element.

Conclusion: Our findings show that the circadian gene *OsLUX* regulates rice heading by directly regulating rhythm oscillation and core flowering-time-related genes. We uncovered a mechanism by which the OsEC target suppresses the expression of *Hd1* and *Ghd7* directly to modulate photoperiodic flowering in rice. The OsEC (OsELF4s–OsELF3-1–OsLUX)–*Hd1*/*Ghd7* regulatory module provides the genetic targets for crop improvement.

Introduction

Nearly 24 h of endogenous rhythm is generated to optimize internal processes that aim to cope with changes in the external environment and provide a fitness advantage for plants [1]. Currently, the most in-depth study about the plant circadian clock is mainly focused on the model plant *Arabidopsis*, particularly in photoperiodic flowering. Flowering time (heading date in crops) is critical for reproductive growth and optimal yield determination in cereals, which is controlled by a sophisticated genetic network including circadian oscillators that coordinate internal physiology and external environmental factors to determine flowering [2]. Thus, how clock genes regulate heading date and improve circadian clock regulation network in rice are topics of great interest.

Generally, the biological clock system consists of three main parts, the input pathway of environmental signal perception, the central oscillator for generating rhythm period, and the output pathway for regulating downstream biological processes [3]. The core oscillator of the plant circadian clock consists of a series of transcription-translation feedback loops (TTFLs) [4]. The EC (evening complex) is composed of the LUX aRRYTHMO (LUX), EARLY FLOWERING 3 (ELF3), and EARLY FLOWERING 4 (ELF4). It is a vital component of maintaining rhythmic oscillation in the evening feedback loops, which releases the suppression effect of pseudo-response regulators (*PRRs*) like *PRR9*, *PRR7*, and *PRR5* on *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) to make sure *CCA1* and *LHY* are expressed in the morning [5–7]. Accordingly, *CCA1* and *LHY* inhibited the expression of the EC complex in the morning, making its expression peak at dusk. Subsequently, it was repressed by *TIMING OF CAB EXPRESSION 1* (*TOC1*) at night [5]. Among different plant species, the EC through LUX binding site (LBS) motif (GATT (A) CG) regulates multiple output pathways, including maintaining circadian rhythms and regulating plant growth and development [5,6,8–10]. For example, mutations in *LUX*, *ELF3*, and *ELF4* caused an arrhythmic and early flowering phenotype in *Arabidopsis* [8,11]. *LUX* orthologs' mutation confers photoperiod-insensitive early heading phenotype in barley and delays flowering in soybean [12,13]. However, whether the OsEC interacts with the photoperiodic flowering pathway to regulate rice heading through multiple flowering output genes like EC in *Arabidopsis* is still unknown. Therefore, further research on OsEC-regulated genes could expand our understanding of OsEC function to regulate heading in rice.

Flowering time acts as a master output pathway of the circadian clock, and the different effects on heading are usually controlled by major circadian components. In rice, numerous flowering-related genes which show rhythmic expression is controlled by the circadian clock. *OsGIGANTEA* (*OsGI*), a critical clock gene, has a rhythmic expression pattern that might regulate circadian clock genes to produce orchestrated rhythms in global transcriptome expression in rice [14]. *Heading date 1* (*Hd1*) is an ortholog of *Arabidopsis CONSTANS* (*CO*), the expression of *Hd1* showed a similar diurnal rhythm pattern under both short-day conditions (SDs) and long-day conditions (LDs) and peaked during the night [15]. *Hd1* works downstream of *OsGI* and promotes heading under SDs while delays heading under LDs by either elevating or repressing *Hd3a* expression [16], which is conservative in the *Arabidopsis GI-CO-FT* pathway. *Grain number*, *plant height*, *and heading date 7* (*Ghd7*) encodes a CCT domain protein, which is a crucial floral repressor in rice under LDs [17,18]. *Ghd7* expression is also controlled by circadian rhythm, and the inducibility peak shifted from dawn under LDs to midnight under SDs. *Early heading date 1* (*Ehd1*) is a critical floral inducer in rice, gate expression induced by blue light in the morning regardless of the day length. However, *Ghd7* suppresses the expression of *Ehd1* and *Heading date 3a* (*Hd3a*) the next morning under LDs. *Ehd1* expression inducibility at dawn under both LDs and SDs, and *Hd3a* expression is acutely induced due to *Ghd7* transcripts being too low to sustain its repressor activity against *Ehd1* at the next dawn under SDs [19].

In addition, previous reports showed that circadian genes are involved in regulating heading in rice. Like *EARLY FLOWERING 3* (*ELF3*) as a floral repressor in *Arabidopsis*, *OsELF3-1*/*Ef7* promotes heading via repressing floral repressor *Ghd7* under LDs [20–22]. Besides, *OsELF3-1* participates in circadian rhythm regulation by suppressing *OsGI*, *OsPRR95*, *OsPRR37*, *OsPRR73*, and *OsPRR1*/*OsTOC1* and promotes the expression of *OsCCA1*/*OsLHY* [21,22]. The *OsPRRs* gene family, the core component of the circadian clock, plays a vital role in regulating photoperiodic flowering in rice, such as *OsPRR37*/*Ghd7.1* delayed heading through negatively regulates the expression of *Ehd1* and *Hd3a* under LDs [23]. *OsPRR73* targeted modulation of floral gene *Ehd1* and the circadian gene *OsLHY* to promote heading [24]. One recent research in rice shows that *OsLHY* sets critical day length for photoperiodic flowering dependent on the *OsGI-Hd1* pathway [25]. Although several major circadian clock genes have been identified to regulate flowering in rice, how the circadian clock interacts with the photoperiodic flowering pathway to regulate rice heading remains to be elucidated.

Previously reported that *OsELF3-1*/*Ef7* mediated flowering through repression of the *Hd1* and *Ghd7* [20]. However, it has yet to be shown via direct evidence of how *OsELF3-1*/*Ef7* achieves to suppress *Hd1* and *Ghd7*. In this study, we cloned the ortholog of *Arabidopsis LUX* using the MutMap method, which acts as a crucial

circadian clock component. *OsLUX* has a positive role in regulating rice heading and might be responsible for the recruitment of OsELF3-1 and OsELF4s to form trimer complex (OsEC) to directly bind to the LBS in the *Hd1* and *Ghd7* promoter to repress their expression. The OsELF3-1 contributes to the translocation of OsLUX to the nucleus, the cytoplasm-nuclear shuttling mechanism explains the effect of OsELF3-1 on OsLUX, and OsELF4s promote the interaction between OsLUX and OsELF3-1. Mutation in the component of the OsEC complex by the CRISPR system brings about a compromised flowering phenotype with distinct effects, which is due to reducing the suppressive activity of OsEC to *Hd1* and *Ghd7*. Thus, *Hd1*/*Ghd7* repressive complex plays a primary role in suppressing heading. Genetic analyses indicate that *Hd1* and *Ghd7* act downstream of *OsLUX*. Collectively, our results provide the OsEC–*Hd1*/*Ghd7* molecular evidence for involving photoperiodic flowering pathway to mediate heading in rice.

Materials and methods

Plant materials and growth conditions

The *elh1*, *elh2*, and *elh3* mutants were screened by EMS mutagenesis of *japonica cv* Nipponbare (Nip) and various mutants, including *oslux*, *oself3-1*, *oself4s*, *oslux-1 oself3-1*, and *oself3-1 oself4s* were grown under natural long-day conditions (NLDs, Hangzhou, China), and then grown in natural short-day conditions (NSDs, Hainan, China). Nip and *elh1* were also cultivated in growth chambers under controlled LDs (CLDs, 14 h light, 30 °C/10 h dark, 25 °C) or controlled SDs (CSDs, 10 h light, 30 °C/14 h dark, 25 °C) in growth chambers with a light intensity of 300 μmol m^{-2} s^{-1} and 70 % relative humidity.

Cloning of OsLUX

We performed a Mutmap method to clone the *OsLUX* gene according to the previous report [26]. The *elh1*, *elh2*, and *elh3* mutants were backcrossed with Nip to generate the F_2 segregation population. Randomly selected 40 F_2 plants with extremely late heading for DNA extraction, the equal amount of DNA was mixed to sequence the whole genome, and then clean sequences were acquired for further analysis. The SNP index was calculated for each SNP site, and the high SNP index is probably the candidate mutation site.

Vector construction and transformation

A 5.59-kb Nip genomic DNA fragment containing the *OsLUX* coding region, 2.98-kb upstream region, and 1.89-kb downstream region was inserted into the pCAMBIA1305 vector at the *Eco*RI site and transformed into *elh1* for genetic complementation. The CRISPR/Cas9 knockout vector construction was performed as described previously [27] to generate various mutants, including *oslux*, *oself3-1*, *oself4s*, *oslux-1 oself3-1*, *oself3-1 oself4s*, *oslux-1 ghd7*, and *oslux-3 hd1*. For overexpression, the coding sequence (CDS) of *OsLUX* was inserted into the pCAMBIA2300 vector at the *Sma*I site to generate the p*Actin*::*OsLUX* construct and transformed into *elh1*. The 2.98-kb upstream fragment of *OsLUX* was amplified from Nip genomic DNA and cloned into the pCAMBIA1305 vector between *Eco*RI and *Nco*I sites to generate a *GUS* reporter gene construct transformed into Nip. The full-length CDSs of *OsLUX*, *OsELF3-1*, and *OsELF4s* were fused at the C-terminal with the pYBA 1132 vector at the *NruI* site, and *OsLUX* fused at the *N*-terminal in pYBA 1132 vector at the *Eco*RI site used for subcellular localization. OsLUX and OsELF4s were fused with mCherry at the *Eco*RI site to generate recombinant plasmids used for co-localization. Then, the expression vector was transformed in rice protoplast or *Nicotiana benthamiana* leaves.

RNA extraction and real-time quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from leaves of 50-day-old seedlings under CLDs and 40-day-old seedlings under CSDs using RNAprep pure Plant Kit (Tiangen Biotech Co. ltd., Beijing, China) according to Kit's instructions. RNA reverse transcribed using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (Toyobo Co. ltd., Osaka, Japan) according to the manufacturer's protocol. RT-qPCR was conducted with SYBR premix Ex Taq Kit (Takara Bio, Inc., Kusatsu, Shiga, Japan) according to the operation instructions. The relative mRNA levels of the investigated genes were normalized to *Ubiquitin* (*Os03g0234350*) by a $2^{-\triangle\triangle CT}$ calculation method with two biological and three technical replicates.

Yeast two-hybrid (Y2H) assay

OsLUX, *OsELF3-1*, and *OsELF4s* CDS amplified from Nip cDNA were used as baits and preys. OsLUX was divided into two truncated fragments (amino acids 1–117 and 118–238), and the truncated fragments of OsELF3-1 (amino acids 1–348, 305–519, and 503–760) were obtained from previously described [28]. The bait and prey fragments were cloned into the pGBKT7 vector between *Eco*RI and *Bam*HI sites and the pGADT7 vector at the *Eco*RI site (Clontech, Takara). OsLUX (amino acids 118–238), OsELF4-1, OsELF4-2, and OsELF4-3 were cloned into the pGBKT7 vector, respectively. OsLUX, OsELF3-1, OsELF3-1 (amino acids 1–348), OsELF3-1 (amino acids 305–519), OsELF3-1 (amino acids 503–760), OsELF4-1, OsELF4-2, and OsELF4-3 were cloned into the pGADT7 vector, respectively. The Y2HGold strains containing both BD and AD constructs were incubated on selective media DDO (SD-Leu/-Trp) or QDO (SD-Leu/-Trp/-His/-Ade) for 4 d at 28 °C.

In vitro pull-down assay

The CDS of *OsLUX*, *OsELF4-1*, *OsELF4-2*, *OsELF4-3* were cloned into the pCold TF vector to generate the construct OsLUX-pCold TF, OsELF4-1-pCold TF, OsELF4-2-pCold TF, and OsELF4-3-pCold TF, respectively. The CDS of OsELF3-1 were cloned into pGEX-4 T-1 to generate GST-OsELF3-1, and Glutathione S-transferase (GST) empty vector was used as a negative control. Expression of GST OsLUX-pCold TF, OsELF4-1-pCold TF, OsELF4-2-pCold TF, and OsELF4-3-pCold TF in BL21 competent cells were induced with 0.1 mM IPTG at 14 °C for 16 h, and GST-OsELF3-1 induced with 0.5 M IPTG at 12 °C for 16 h, each combined solution added to 30 μL glutathione high capacity magnetic agarose beads (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation at room temperature for one hour with rocking. The beads were washed four times with pull-down buffer (50 mM/L Tris-HCl, pH 7.5, 5 % glycerol, 1 mM/L EDTA, 1 mM/L DTT, 1 mM/L PMSF, 0.01 % Nonodet P-40, and 150 mM/L KCl), and the proteins were separated on SDS-PAGE gels and detected by anti-GST antibody (TransGen Biotech Co. ltd., Beijing, China; lot number HT601, 1:5000 dilution) and anti-His antibody (TransGen Biotech; lot number HT501-01, 1:5000 dilution), respectively.

BiFC assay

OsLUX and *OsELF4s* cDNAs were cloned into 35S-SPYNE and 35S-SPYCE vectors, respectively, named OsLUX-YN, OsELF4-1YC, OsELF4-2YC, and OsELF4-3YC. In addition, the complete CDS of *OsELF3-1* was cloned into pCAMBIA1305-mcherry. The constructs were transformed into *Agrobacterium* strain GV3101 (pSoup) and

then co-transfected into *Nicotiana benthamiana* leaves of 3-week-old. The mcherry was used as a control.

Cell fractionation assays

The cellular components were isolated according to the previous description [29]. The cDNA fragments of the *OsLUX* and *OsELF3-1* genes were fused to the N terminus of HA and C terminus of GFP, respectively. Transfection of the HA and GFP expression vector into rice protoplasts. Collecting the cultured cells at 36 h later and then suspended in lysis buffer (20 mM Tris-HCl, PH7.5, 20 mM KCl, 2 mM $MgCl_2$, 25 % Glycerol, 250 mM sucrose, 50 mM DTT) at 4 °C, and centrifuged at 12000 rpm for 10 min, the resulting supernatant is total protein. The resultant supernatant was first centrifuged at 1500 g for 10 min, then centrifuged at 10,000 g for 10 min. The resultant supernatant was the soluble cytosol fraction, and the crude pellet was the nucleus fraction. The crude nucleus fraction was resuspended in 1 ml of washing buffer (20 mM Tris-HCl, PH 7.5, 25 mM $MgCl_2$, 25 % Glycerol, 0.2 % Triton X-100). It was centrifuged at 15,000 g for 10 min and repeated the operation several times. After the washing step was finished, the pellet was diluted with 500 μL resuspend buffer (20 mM Tris-HCl, PH 7.5, 10 mM $MgCl_2$, 250 mM sucrose, 0.5 % Triton X-100, 5 mM β-Mercaptoethanol) and transferred to another tube with the buffer (20 mM Tris-HCl, PH 7.5, 1.7 M sucrose, 10 mM $MgCl_2$, 0.5 % Triton X-100, 5 mM β-Mercaptoethanol), and then centrifuged at 16,000 g for 45 min, the final pellet was the nucleus protein and resuspended with lysis buffer.

Luciferase complementation assay (LCA)

The CDSs of the *OsLUX* and *OsELF4s* were cloned into the N terminus of luciferase (LUC) in the pCAMBIA-nLUC vector to generate OsLUX-nLUC, OsELF4-1-nLUC, OsELF4-2-nLUC, OsELF4-3-nLUC constructs, respectively. Similarly, OsLUX-cLUC and OsELF3-1-cLUC were developed by the pCAMBIA-cLUC vector, which fused to the C terminus of LUC. The *Agrobacterium* strain GV3101 (pSoup) carried with nLUC and cLUC recombinant plasmids were co-transfected into *Nicotiana benthamiana* leaves through equally mixed. After 48 h infiltration, *Nicotiana benthamiana* leaves were injected with 1 mmol/L D-luciferin potassium substrate for the qualitative detection of luciferase activity by a charge-coupled device (CCD) imaging system. Leaf discs were incubated with 200 μL of 1 mM μL^{-1} D-luciferin potassium in a 96-well plate for quantitative detection of luciferase activity with a GloMax 96 microplate luminometer (Promega, Madison, WI, USA).

Yeast one-hybrid assay

The CDS of *OsLUX* and the promoter of *OsPRRs* were cloned into pB42AD and pLacZi reporter vectors, respectively. The EGY48 strains containing both pB42AD and pLacZi constructs were incubated on SD/-Ura/-Trp plates, and then grew on SD/-Ura/-Trp with 1 × BU salts, 1 % raffinose, 2 % galactose, and 80 mg/L X-Gal. The blue colonies indicate the interaction.

Electrophoretic mobility shift assay (EMSA)

The BL21 (DE3) strain (Tsingke Biotech Co. ltd., Beijing, China) was carried with a GST-OsLUX recombinant construct and expressed with 0.1 mM IPTG (isopropyl-1-thio-D-galactopyranoside) at 14 °C for 12 h, and then using Beaver Beads™ GSH (Beaver Biosciences Inc, Suzhou, China; catalog no. 70601–100) to purify the GST-OsLUX fusion protein. The probes containing LBS motifs in *Hd1* and *Ghd7* promoter were synthesized and labeled with biotin at the 3′-end by the EMSA Probe Biotin Labeling Kit (Beyotime Institute of Biotechnology, Shanghai, China). The EMSA experiment was conducted using the Chemiluminescent EMSA Kit (Beyotime) according to the manual provided by the manufacturer. The labeled probes or unlabeled oligonucleotides were incubated with GST-OsLUX fusion protein in 10 μL mixtures at 23 °C for 30 min. The mixtures were separated with 6 % polyacrylamide gels and visualized using a chemiluminescence imaging system (Bio-Rad Laboratories, Inc, Segrate, Italy).

Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) assay

The ChIP-qPCR assay was performed as described [30]. About 3 g leaf tissue of 35S::3 × flag:OsLUX seedlings with 30-day-old under NLDs was used for crosslinking fixation. Chromatin was fragmented to 200–700 bp by sonication. The antibody used for ChIP-grade antibody against FLAG (Sigma-Aldrich, St. Louis, MO, USA; catalog no. F1804). The input and precipitated DNA samples were used as a template for performing qPCR. The values were normalized to input samples, and IgG was used as a negative control.

Luciferase transient transcriptional activity assay

The CDS of *OsLUX* was inserted into the pGreenII 62-SK vector at the *Bam*HI site, and the promoters of *Hd1* and *Ghd7* were cloned into the reporter vector pGreenII 0800-LUC at the *Hind*III site. The *Agrobacterium* strain GV3101 (pSoup) carried with effector and recombinant reporter plasmid were co-transformed into *Nicotiana benthamiana* cells. A Dual-Luciferase® Reporter Assay System (Promega) was used to measure the firefly and renilla LUC signals according to the manufacturer's instructions. The data was indicated as mean ± SD, and each assay was performed with three biological and three technical replicates. In addition, full-length *OsLUX*, *OsELF3-1*, and *OsELF4s* cDNA were inserted into pYBA 1132 to generate the GFP–OsLUX, GFP–OsELF3-1, and GFP–OsELF4s constructs, and then co-transfected into *Nicotiana benthamiana* leaves with *Hd1*–LUC or *Ghd7*–LUC to achieve luciferase luminescence images.

Results

elh1/oslux-1 mutant exhibits extremely late heading phenotype

To identify new rice genes regulating heading, we screened an extremely late heading mutant, *elh1*, from our rice mutant library generated by ethyl methanesulfonate-induced in Nipponbare (Nip). The mutant displayed 63.2 days later heading than Nip under NLDs at Hangzhou and 76.6 days later under NSDs at Hainan (Fig. 1A-B). In addition, we grew the *elh1* mutants and Nip plants in controlled chambers under CLDs and CSDs. Under CSDs, the heading date of the *elh1* mutants (214.7 ± 19.2 d) was delayed by 160.7 d compared with the Nip (54.0 ± 2.7 d). Under CLDs, Nip plants headed at 80.3 ± 5.0 d; however, the *elh1* mutants exhibited no heading for more than 260 d (Fig. 1B). The extremely late heading phenotype of the *elh1* mutants under both photoperiodic conditions suggests that this mutant significantly affected heading. The leaf emergence rates of *elh1* had no difference with Nip (Fig. S1-A-B), indicating that the delayed heading in the *elh1* mutants was due to prolonged floral transition but not retarded growth under both CLDs and CSDs. Moreover, under Hangzhou NLDs, we found the *elh1* mutants showed shorter panicles than the Nip, but it's the other way around under NSDs at Hainan. In addition, the primary branches of *elh1* mutants were significantly increased while the grain size and thousand-grain weight are markedly smaller and lighter than Nip (Fig. S1, table. S1).

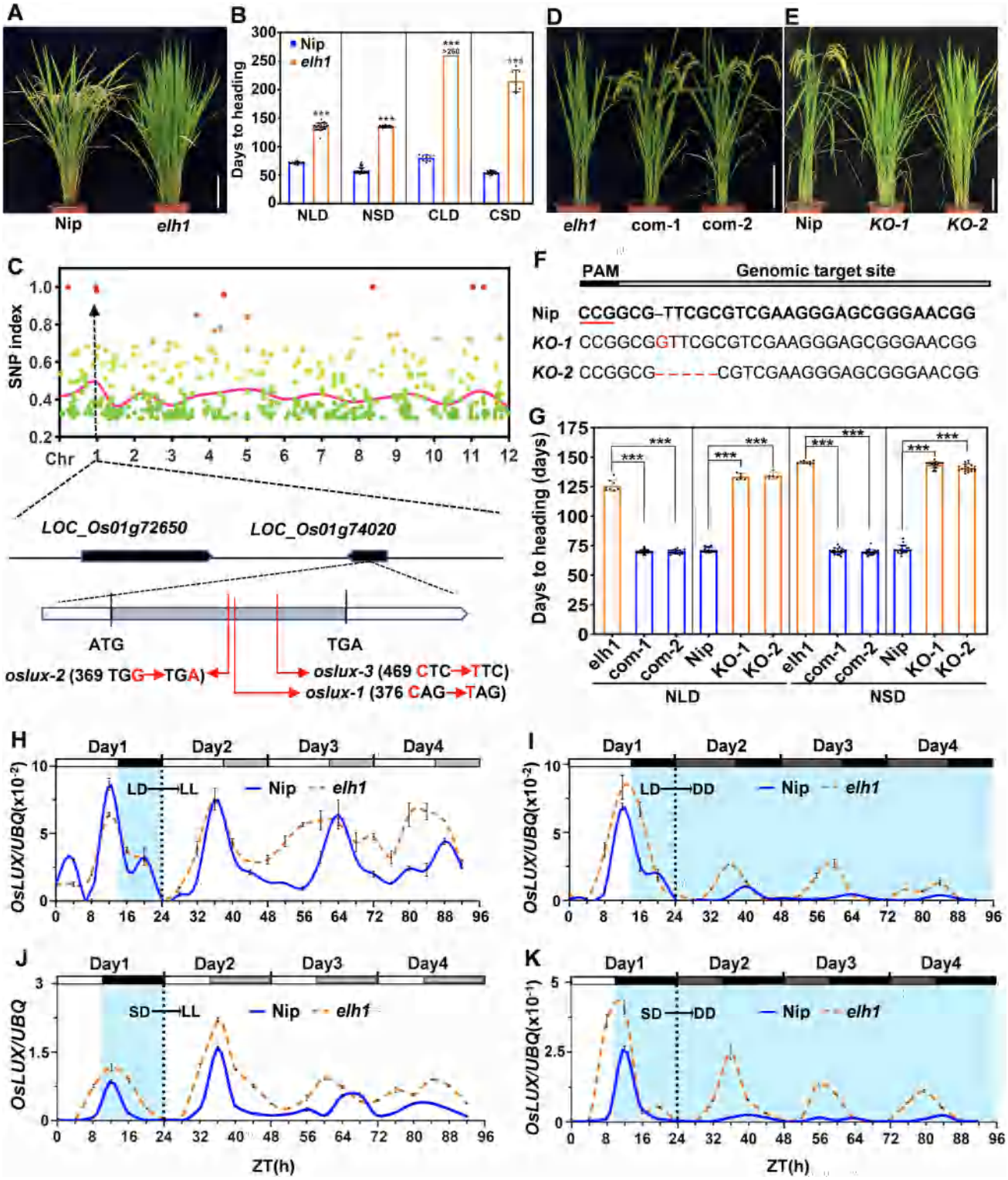

Fig. 1. The role of *OsLUX* in rice heading. (A) Phenotypes of the mature period with Nip and *elh1* under NLD conditions in Hangzhou. Bar = 20 cm. (B) Heading data of Nip and *elh1* under NLD, NSD, CLD, and CSD conditions. The asterisk indicates statistically significant differences with $P < 0.001$. (C) The identification of *oslux* alleles. The Top section indicated distributions of SNP indexes along chromosomes in *elh1*. *OsLUX* gene structure and allelic mutation of *oslux-1*, *oslux-2*, and *oslux-3* are shown in the bottom section; red characters indicate the mutant site. (D) Heading phenotypes of Nip and the *elh1*-complementation lines under NLDs, Scale bar = 20 cm. (E) The phenotype of Nip and two independent knockout lines under NLDs, Scale bar = 20 cm. (F) mutation sites of the CRISPR-edited sgRNA target sites of *OsLUX*, red underline indicate PAM sequence. (G) The heading date investigation of complementation lines and knockout lines in both NLDs and NSDs. The asterisk indicates statistically significant differences with $P < 0.001$. (H, I) Diurnal expression patterns of *OsLUX* from LD to LL and LD to DD, respectively. (J, K) Diurnal expression patterns of *OsLUX* from SD to LL and SD to DD, respectively. The blue and white boxes indicate dark and light periods, respectively. ZT, Zeitgeber time, ZT = 0 represents the start-time of lights on. The error bars represent standard deviations.

OsLUX encodes a transcription factor that belongs to the MYB family

We then performed cloning of the *elh1* mutant gene using the MutMap method. The *elh1* mutant first back-crossed with the Nip, and all F_1 progeny displayed normal heading. The F_2 population exhibited 3:1 segregation of normal heading and extremely late heading (176:55, χ^2 = 0.17, P = 0.68), indicating that *elh1* is a single recessive mutation. The MutMap analyses indicated a candidate region at the end of chromosome 1, including three candidate SNPs: one of which is in the intergenic regions, one in the intron of *LOC_Os01g72650*, and one (Chr1: 42,874,923) in the exon of *LOC_Os01g74020* which resulted in a stop-gain mutation (CAG to TAG) at position 376 (Fig. 1C). Co-segregation analysis showed that all 55 extremely late heading plants carried this mutation, whereas the 176 normal heading plants showed a 2:1 ratio of heterozygous and homozygous Nip genotypes (117:59). *LOC_Os01g74020* putatively encodes an MYB family circadian clock gene *OsLUX* (also known as *OsPCL1*), an ortholog of *Arabidopsis LUX* [31,32], and *OsLUX* is highly orthologous among both monocot and dicot species. *OsLUX* is a single-copy gene closely related to maize *ZmLUX* (Fig. S2A) and encodes a transcription factor with a conserved MYB DNA binding domain in the C terminus (Fig. S2B).

To verify the function of *LOC_Os01g74020*, genetic complementation assay was carried out with a 5.59-kb genomic fragment from Nip to introduce into the *elh1*/*oslux-1* mutant, and positive transgenic plants were able to fully rescue the extremely late heading phenotypes under both NLDs and NSDs (Fig. 1D, 1G). When a p*Ubi*::*OsLUX* construct was transferred to the *elh1* mutant, the transgenic plants ectopically expressed *OsLUX* showed a normal heading phenotype (Fig. S3A-D). The *oslux* mutants generated by CRISPR/Cas9 exhibited a similar phenotype to the *elh1* mutant under both NLDs and NSDs (Fig. 1E-G), suggesting that the mutation in *LOC_Os01g74020* is responsible for the extremely late heading phenotype of *elh1*. In addition, we evaluated two other mutant alleles of *LOC_Os01g74020* (*elh2*/*oslux-2* and *elh3*/*oslux-3*, Fig. 1C). The *elh2*/*oslux-2* also causes a stop-gain mutation (TGG to TGA) at position 369, which had an exceptionally late heading phenotype similar to *elh1*. In contrast, the *elh3*/*oslux-3* causes amino acid substitution at position 469 (CTC to TTC), which resulted in Leu to Phe, and exhibited 34.4 days and 36.9 days later heading compared to Nip under NLDs and NSDs, respectively (Fig. S4A-B). Taken together, these results confirmed the positive role of *OsLUX* in rice flowering time regulation.

OsLUX has a constitutive and rhythmic expression pattern

To understand the spatio-temporal expression pattern of *OsLUX*, we first performed RT-qPCR to examine the mRNA levels of *OsLUX* under NLDs from root, stem, leaf blade, and leaf sheath at the vegetative stage and the different lengths of young panicles at the reproductive stage. The results revealed that *OsLUX* was constitutively expressed among different tissues preferentially accumulated in leaf blades, which is in accord with a β-glucuronidase (GUS) activity assays shown by expressing the p*OsLUX*::GUS (Fig. S5A-B). Next, we examined the diurnal expression pattern. The results showed that *OsLUX* transcripts were increased during the light period and reached a peak at ZT = 12, subsequently reduced to the lowest level at dawn under LDs and SDs. However, the rhythm and amplitude of *OsLUX* transcripts were slightly decreased when transferred to continuous light (LL) conditions (Fig. 1H, 1 J). Still, they declined sharply when shifted to continuous dark (DD) conditions (Fig. 1I, 1 K).

Moreover, the expression waveform or amplitude of *OsLUX* was slightly higher in *elh1* than Nip, probably due to the negative feedback regulation of *LUX*, as shown in *Arabidopsis* [6,9]. In fact, we found the native *OsLUX* transcript levels were strongly reduced in 35S::3 × flag:OsLUX (Fig. S5C), which showed that *OsLUX* participates in a feedback loop. Together, these results revealed that *OsLUX* acts as a circadian gene necessary for photoperiodic response to regulate heading in rice.

OsLUX is involved in maintaining the circadian rhythm and regulating heading in rice

The flowering time/heading date is strictly regulated by the circadian rhythm clock. Thus, we examined the rhythm expression of the clock genes in rice. The results showed that the expression levels of *OsPRR37*, *OsPRR73*, *OsPRR59*, *OsPRR95*, and *OsPRR1* were up-regulation in *elh1* and resulted in a higher amplitude of the rhythmical pattern compared to Nip under normal photoperiod. Still, in LL conditions, the expression patterns of these genes showed an arrhythmic oscillation (Fig. S6A-E, 6G-K). In contrast, the transcriptional levels and amplitude of *OsCCA1* were significantly lower than Nip under regular photoperiod. When transferred to LL conditions, the amplitude of *OsCCA1* in Nip and *elh1* decreased sharply (Fig. S6F, 6L). To explore whether OsLUX bind to the promoter of these circadian clock gene, we performed a yeast one-hybrid assay. We found the promoter of clock genes with potential LBS motifs except for *OsCCA1*/*OsLHY*. The results showed that OsLUX activated the *LacZ* expression of *OsPRR1*, *OsPRR59*, and *OsPRR95* but not *OsPRR37* and *OsPRR73* (Fig. S7D), which indicated that OsLUX might directly bind to the promoter of *OsPRR1*, *OsPRR59*, and *OsPRR95* to regulate their expression. Collectively, these results showed that *OsLUX* is involved in maintaining the circadian clock of rice.

To evaluate how *OsLUX* regulates the rice flowering pathway, the expression levels of flowering-time-related genes were also monitored in Nip and *elh1* mutants via RT-qPCR under LDs and SDs. We first examined two floral integrators, *Hd1* and *Ehd1*. *Hd1* transcripts were up-regulated in *elh1* mutants compared with Nip (Fig. 2A-B). However, *Ehd1* transcripts were almost undetectable in *elh1* mutants (Fig. 2C-D), indicating that *OsLUX* represses *Hd1* and promotes *Ehd1* expression. Subsequently, we examined the expression levels of *Hd3a* and *OsMADS14*, which lie downstream of *Hd1* and *Ehd1*. These genes showed similar expression patterns to *Ehd1* (Fig. 2E-H), indicating that *OsLUX* plays a vital role in flowering initiation. Finally, we investigated the transcripts of *OsGI* and *Ghd7*, which are upstream regulators of *Hd1* and *Ehd1*, respectively. Notably, their transcripts significantly increased in *elh1* mutants (Fig. 2I-L). Our results were similar to a previous report that the overexpression of *OsGI* caused extremely late heading phenotype through up-regulate *Hd1* transcription levels under both LDs and SDs [33]. These results suggest that *OsLUX* promotes heading by suppressing *OsGI*, *Hd1*, and *Ghd7*.

OsLUX forms a heterotrimer complex through the recruitment of OsELF3-1 and OsELF4s

To further investigate the molecular mechanism underlying *OsLUX* regulates heading, we explored whether rice forms an EC complex similarly to *Arabidopsis*. In rice, there are three orthologs of *AtELF4*: *OsELF4-1* (*LOC_Os11g40610*), *OsELF4-2* (*LOC_Os03g29680*), and *OsELF4-3* (*LOC_Os08g27860*), and two orthologs of *AtELF3*: *OsELF3-1* (*LOC_Os06g05060*) and *OsELF3-2* (*LOC_Os01g38530*). *OsELF3-1* is involved in circadian rhythm regulation and promotes heading [21,28], while *OsELF3-2*'s predominant role is immunity regulation [34]. Consequently, we focused on whether *OsELF3-1* mediates flowering through the rice EC complex.

We conducted yeast two-hybrid (Y2H) assays to test the physical interactions between OsLUX with OsELF3-1 and OsELF4s. Due to the autoactivations activity of OsLUX, truncated fragments of

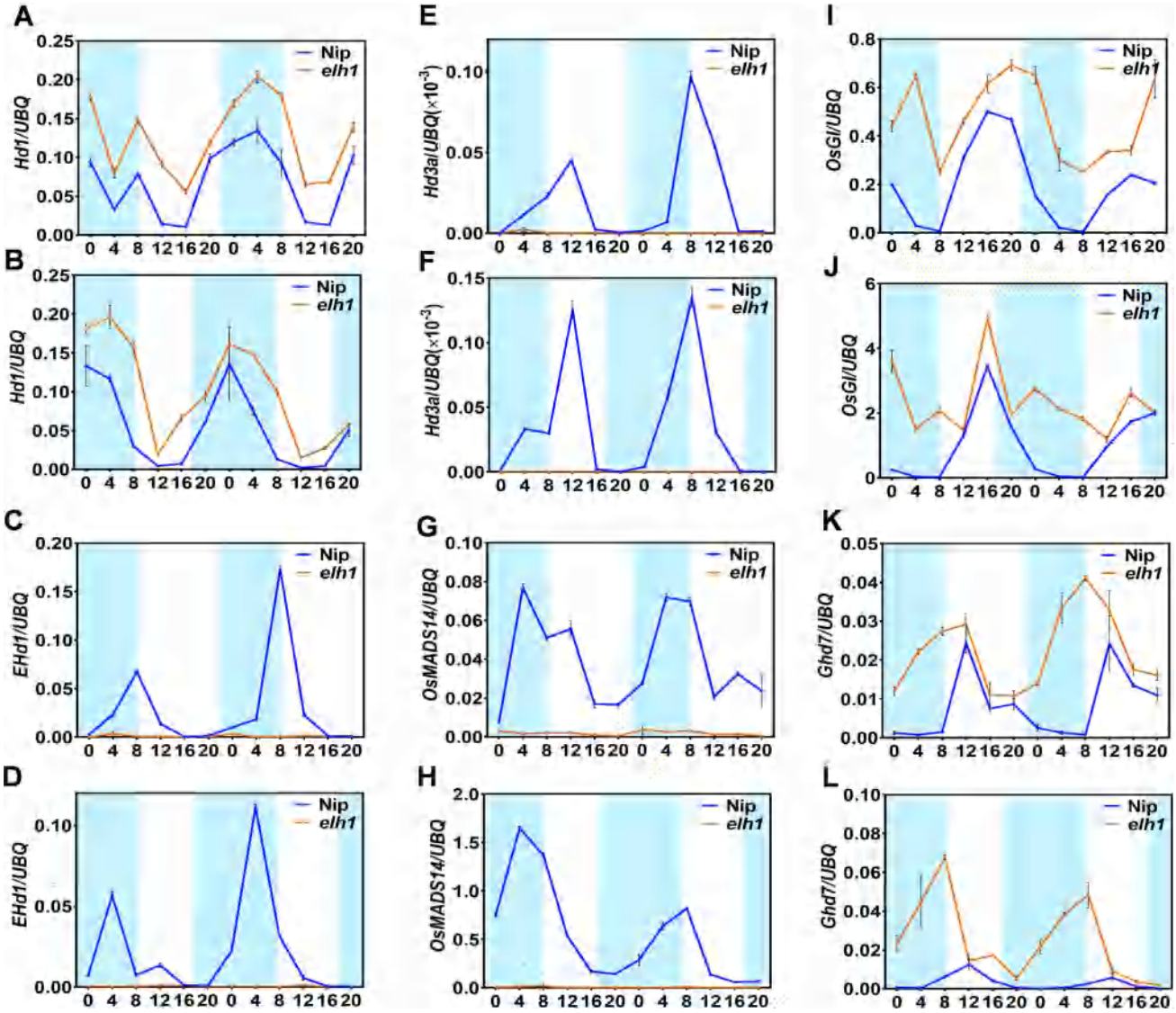

Fig. 2. Diurnal expression of flowering-time-related genes under LDs (14 h light, 10 h darkness) and SDs (10 h light, 14 h darkness) in Nip and *elh1*. The blue and white filled boxes indicate dark and light periods, respectively. The samples were collected every 4 h under both LDs and SDs. The relative expression levels of *Hd1* (A, B), *Ehd1* (C, D), *Hd3a* (E, F), *OsMADS14* (G, H), *OsGI* (I, J), and *Ghd7* (K, L) normalized with rice *Ubiquitin* under LDs and SDs, respectively. The Zeitgeber time (ZT). ZT = 8 is set to the time of lights on.

OsLUX (amino acids 118–238) with no transcriptional activation activity (Fig. S7). Through Y2H assay, we found that the C-terminal domain (amino acids 503–760) and the middle region (amino acids 305–519) of OsELF3-1 could mediate a physical interaction with OsLUX and OsELF4s, respectively. However, OsLUX and OsELF4s failed to interact with each other (Fig. 3A). The interaction was also supported by pull-down (Fig. 3B) and luciferase complementation assays (LCA; Fig. S8). We performed another LCA and found that the LUC signal can be generated between OsLUX-Cluc with OsELF4s-Nluc due to the existence of mCherry-OsELF3-1 rather than mCherry alone (Fig. 3C-E). Collectively, these results confirmed that OsELF3-1 acts as a bridge to allow the formation of the ternary complex (OsELF4s-OsELF3-1-OsLUX).

OsELF3-1 contributes to the translocation of OsLUX to the nucleus

The nuclear localization of the OsEC complex is vital for the transcriptional regulation of target genes. To investigate the subcellular localization of OsLUX, a transient expression assay was performed using tobacco cells, showing that the p*35S*::OsLUX:GFP fusion protein was located in both the cytosol and the nucleus, which the same results were confirmed with p*35S*::GFP:OsLUX transient expression in tobacco cells and rice protoplasts (Fig. 4A; Fig. S9). Consistent with previous reports [21,35], we also conducted the subcellular localization of OsELF3-1 and OsELF4s and found that they only localized to the nucleus (Fig. S9). To exam whether OsELF3-1 affects the translocation of OsLUX to the nucleus, we transiently co-expressed p*35S*::OsLUX:mCherry and p*35S*::GFP:OsELF3-1 in rice protoplasts. The fusion signal was detected only in the nucleus but not in the cytoplasm, whereas, OsLUX-GFP co-expressed with mCherry were co-localized to both the cytoplasm and nucleus (Fig. 4A). This observation shows that OsELF3-1 alters the subcellular localization of OsLUX and promotes OsLUX translocation into the nucleus. The cell fractionation and immunoblotting assays in rice protoplasts also confirmed that OsLUX was detected in the cytosol and nucleus fraction (Fig. 4C), and the OsELF3-1 was present in the nucleus fraction (Fig. 4D). We co-expressed OsLUX and OsELF3-1 in rice protoplasts and found both OsLUX and OsELF3-1 had accumulated in the nucleus fraction (Fig. 4E).

Bimolecular fluorescence complementation (BiFC) assay was conducted to detect the subcellular location of the OsLUX–OsEL

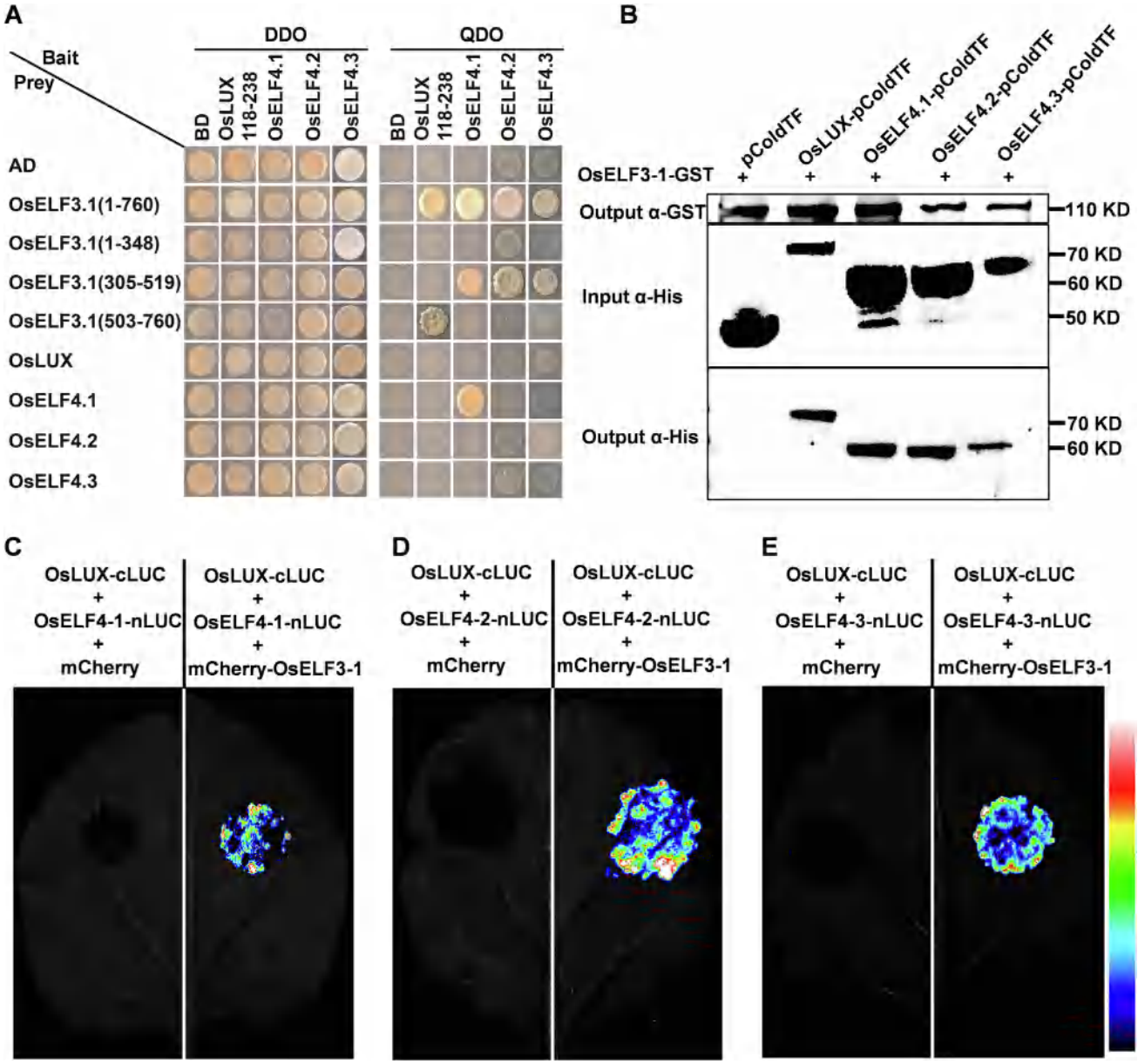

Fig. 3. OsELF3-1 interacts with OsLUX and OsELF4s *in vivo* and in *vitro*. (A) Yeast two-hybrid assay of interactions between OsELF3-1 with OsLUX and OsELF4s. OsELF3-1 and its truncated fragments as prey with bait constructs, including OsELF4s and OsLUX truncated fragments, the bait and prey constructs were cotransformed into Y2H Gold yeast cells and then grown on DDO medium (SD–Leu/–Trp) and QDO medium (SD–Leu/–Trp/-His/-Ade). (B) A pull-down assay demonstrated that OsELF3-1 directly interacts with OsLUX and OsELF4s. pCold-TF-OsLUX and pCold-TF-OsELF4s were pulled down by GST-OsELF3-1 immobilized on glutathione Magnetic Agarose beads and analyzed by immunoblotting (IB) with anti-His antibody. (C) The luciferase complementation assays (LCA) validation of OsELF3-1 is required for ternary complex formation with OsLUX and OsELF4s. mCherry was used as the negative control.

F3-1–OsELF4s interaction, and the fluorescence signals were observed only in the nucleus when expressing OsLUX–YFP^N/OsELF4s–YFP^C due to the existence of mCherry–OsELF3-1 compared to mCherry alone (Fig. 4B). OsLUX failed to interact with OsELF4s, which triggered us to consider the effects of OsELF4s on OsLUX–OsELF3-1 interaction. In *Arabidopsis*, ELF4 promotes the nuclear localization of ELF3 [36]. Our results found that OsELF3-1 contributes to the translocation of OsLUX to the nucleus (Fig. 4A, 4C-E), so we want to know if OsELF4s might promote the interaction between OsLUX and OsELF3-1. We used LCA assays to test this hypothesis; the relative luciferase activity observed for the OsELF3-1–OsLUX interaction was significantly increased when OsELF4s were co-expressed with OsELF3-1 and OsLUX (Fig. S10). Taking the results from co-localization, BiFC, and cell fractionation immunoblotting assays together, we conclude that OsELF3-1 contributes to the translocation of OsLUX to the nucleus and the localization of the OsEC complex in the nucleus, and complete OsEC activity is necessary for regulating output genes.

Defect in OsEC causes compromised heading

To verify the genetic relationship between the OsLUX–OsELF3-1–OsELF4s trimer components, CRISPR/Cas9 was used to generate null mutants for each of the three genes in the Nip background, and *oslux-1 oself3-1* and *oself3-1 oself4s* double mutants were also made. Consistent with previous reports [20], the mutation in *OsELF3-1* resulted in 22.1-d and 18.2-d later heading than Nip under NLDs and NSDs, respectively (Fig. 5A-B). Under NSDs at Hainan, we obtained the *oslux-1 oself3-1* double mutant through screening F_2 plants from the hybridized *elh1* and *oself3-1*. The

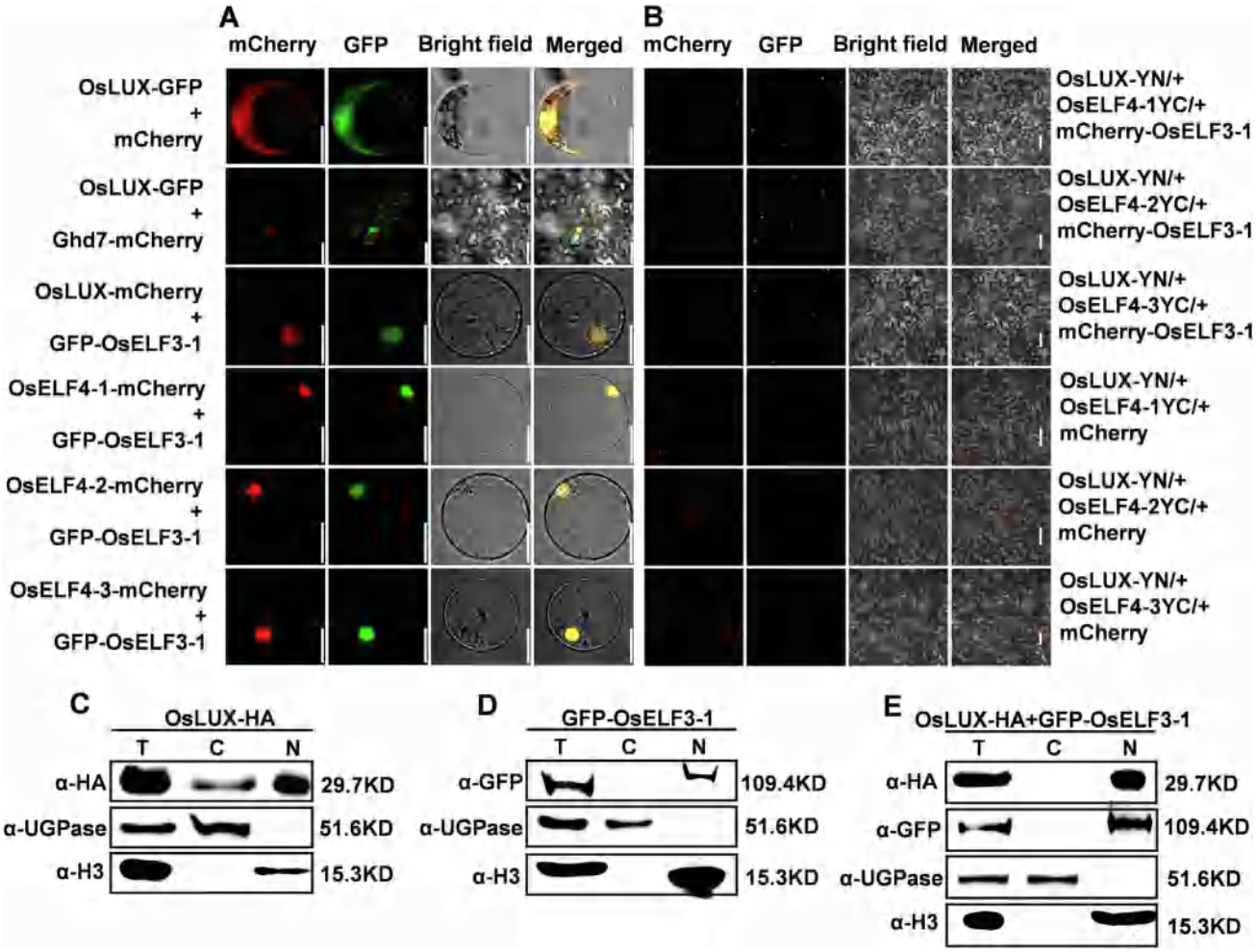

Fig. 4. The co-localization and BiFC of OsELF3-1 between OsLUX and OsELF4s. (A) Analysis of the subcellular localization of OsLUX-GFP using rice protoplasts and *Nicotiana benthamiana* cells transient expression, and co-localization of OsELF3-1 between OsLUX and OsELF4s using rice protoplast. Ghd7-mCherry is used as a nuclear marker. (B) OsELF3-1 interacts with OsLUX and OsELF4s confirmed by bimolecular fluorescence complementation (BiFC) assay, OsLUX-YN and OsELF4s-YC were co-expressed with mCherry or mCherry-OsELF3-1 in tobacco epidermal cells. mCherry as a control, Bar = 10 μm. (C-E) The cell fractionation and immunoblotting assays in rice protoplasts. (C) OsLUX, (D) OsOsELF3-1, (E) both OsLUX and OsOsELF3-1 in cell fractions extracted from rice protoplasts. T, total protein, C, cytosol protein, N, nucleus fraction. The UGPase (cytoplasm marker) and H3 (nucleus marker) were used in immunoblotting.

oslux-1 oself3-1 double mutant resembled the *elh1* mutant, exhibiting an extremely late heading phenotype. (Fig. 5G-H;). As for the *elh1* mutants, a recent study reported that *oself4-1* delayed heading in Beijing NLDs [35], while in our study, the mutation in *OsELF4-2* (but not *OsELF4-1* and *OsELF4-3*) resulted in late heading in Hangzhou NLDs (Fig. 5C-D; Fig. S11A-D). Under Hainan NSDs, the *elf4-1* has no difference in heading, but the *elf4-2* and *elf4-3* mutants flowered slightly later than Nip (Fig. 5D; Fig. S11D). The late heading phenotype of *oself3-1 oself4-2* plants resembles the *oself3-1* single mutant under NSDs (Fig. 5I-J). Our results suggest that *OsELF4s*, which have a minor effect on rice heading, may function together to form a ternary complex with OsELF3-1 and OsLUX to regulate photoperiodic flowering. Like EC mutants display the early flowering in *Arabidopsis*, a compromised flowering phenotype results upon mutation of any component of the OsEC complex.

OsEC complex binds to the promoters of Hd1 and Ghd7 and suppresses their expression

Hd1 and *Ghd7* have been reported as crucial regulators of photoperiodic flowering in rice. The expression levels of *Hd1* and *Ghd7* were up-regulated, and similar expression patterns under both LDs and SDs in *elh1* mutants (Fig. 2A-B, K-L). In *Arabidopsis*, LUX binds to the LBS motifs in the promoters of the output genes [9,37]. We found putative LBS motifs in the promoter of *Hd1* and *Ghd7* via using the online tool PlantCARE (Plant cis-Acting Regulatory Elements, https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [38], which implied that *Hd1* and *Ghd7* might be the direct target genes of *OsLUX*. To validate this hypothesis, Chip-qPCR analysis was performed using p*35S*::FLAG:OsLUX expressing seedlings with anti-FLAG antibodies under NLDs. Four primer pairs were designed within 2-kb upstream of the transcription initiation site, which corresponds to four putative LBS motifs (S1, S2, S3, and S4, Fig. 6A), and significant enrichment of OsLUX on the S4 region *in vivo* was found instead of S1 S2 and S3 (Fig. 6B). We also conducted an electrophoresis mobility shift assay (EMSA) further to confirm this result, glutathione S-transferase (GST)-tagged OsLUX fusion protein bound to the S4 site in the *Hd1* promoter with a retarded band, when fusion protein incubated together with the unlabeled oligonucleotide and the shifted band was substantially weakened with unlabeled in a dosage-dependent manner (Fig. 6D), indicating that OsLUX bind to the S4 site in particular. Moreover, a transcriptional activity assay was carried out further to confirm the suppression effect of OsLUX to *Hd1*. The p*35S*::*OsLUX* expression construct was co-transformed with p*Hd1*::LUC into *Nicotiana benthamiana* leaves, and the results showed the rel-

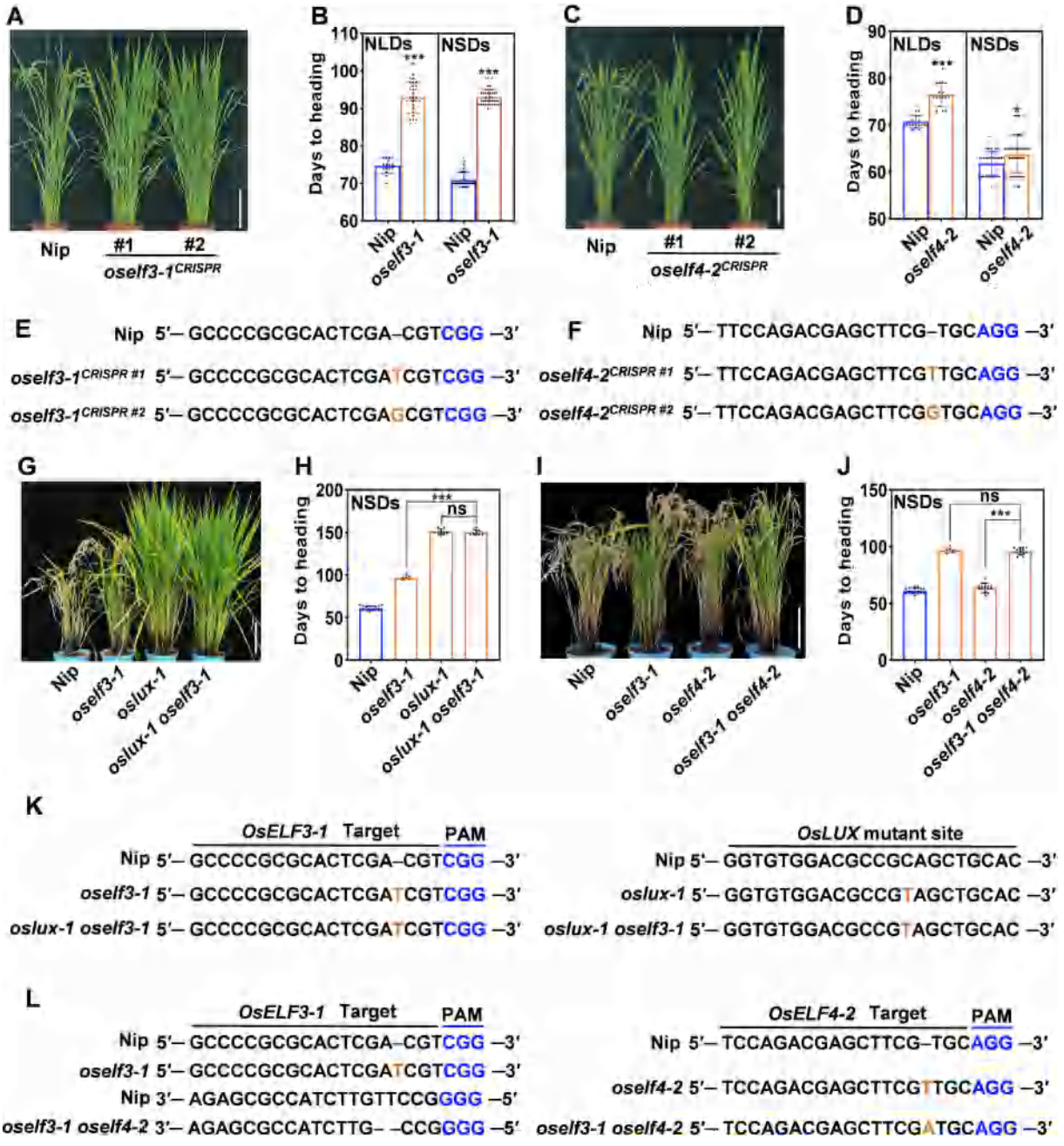

Fig. 5. The phenotype of the *oself3-1*, *oself4-2*, and *oslux-1 oself3-1*, *oself3-1 oself4-2* double mutants under different photoperiod conditions. (A and B) The phenotype (A) and days to heading (B) of Nip and *oself3-1* lines. (C and D) The phenotype (C) and days to heading (D) of Nip and *oself4-2* lines. The photos in A and C were taken under NLDs in Hangzhou, and the data from B and D were obtained under both NLDs and NSDs. (E and F) The mutant site of CRISPR-edited *oself3-1* (E) and *oself4-2* (F). (G and H) The phenotype (G) and days to heading (H) of Nip, *oself3-1*, *oslux-1*, and *oslux-1 oself3-1* under NSDs in Hainan. (I and J) The phenotype (I) and days to heading (J) of Nip, *oself3-1*, *oself4-2*, and *oself3-1 oself4-2* under NSDs in Hainan. (K and L) The genotype of mutants at *OsELF3-1* and *OsLUX* sites (K) and *OsELF3-1* and *OsELF4-2* sites (L). The blue characters indicate the PAM sequence, and the tangerine character indicates the mutant site. All statistical data represent mean ± SD. *** indicates statistically significant differences with $P < 0.001$ from student's *t*-test, * indicates $P < 0.05$. Bar = 20 cm.

ative LUC activity in p*35S*::*OsLUX* was significantly lower than the negative control (Fig. 6C, F). Similarly, OsLUX also directly binds to the LBS in the *Ghd7* promoter to suppress its expression (Fig. 6A, E, G). Genetic studies further corroborated the biological function of *OsLUX* in *Hd1* and *Ghd7* regulation. We obtained the *oslux-3 hd1* double mutant through generating a null mutation of *Hd1* by CRISPR/Cas9 (Fig. 7E) against the background of *elh3*, and the *oslux-1 ghd7* double mutants via screening F_2 plants from the F_1 (*elh1* × *ghd7*, Fig. 7F). Knock out *Hd1* and *Ghd7* neutralized the repression of *oslux* on rice heading, which the *oslux-3 hd1* and *oslux-1 ghd7* double mutants restored the extremely late heading phenotype of the *elh3* and *elh1*, respectively (Fig. 7A-B, 7C-D). The genetic data suggest that *Hd1* and *Ghd7* act downstream of *OsLUX* to regulate rice flowering.

To determine the effects of the OsEC complex on transcriptional repression of *Hd1* and *Ghd7*, we performed a LUC transient transcriptional activity assay. When a p*35S*::*OsLUX* expression construct was co-transformed into *Nicotiana benthamiana* leaves with p*Hd1*::*LUC*, the luciferase luminescence was significantly suppressed compared with the negative control (Fig. S12A-C), indicating that OsLUX acts as a transcription repressor to bind to suppress *Hd1* activity. When a p*35S*::*OsELF3-1* construct was added, the luci-

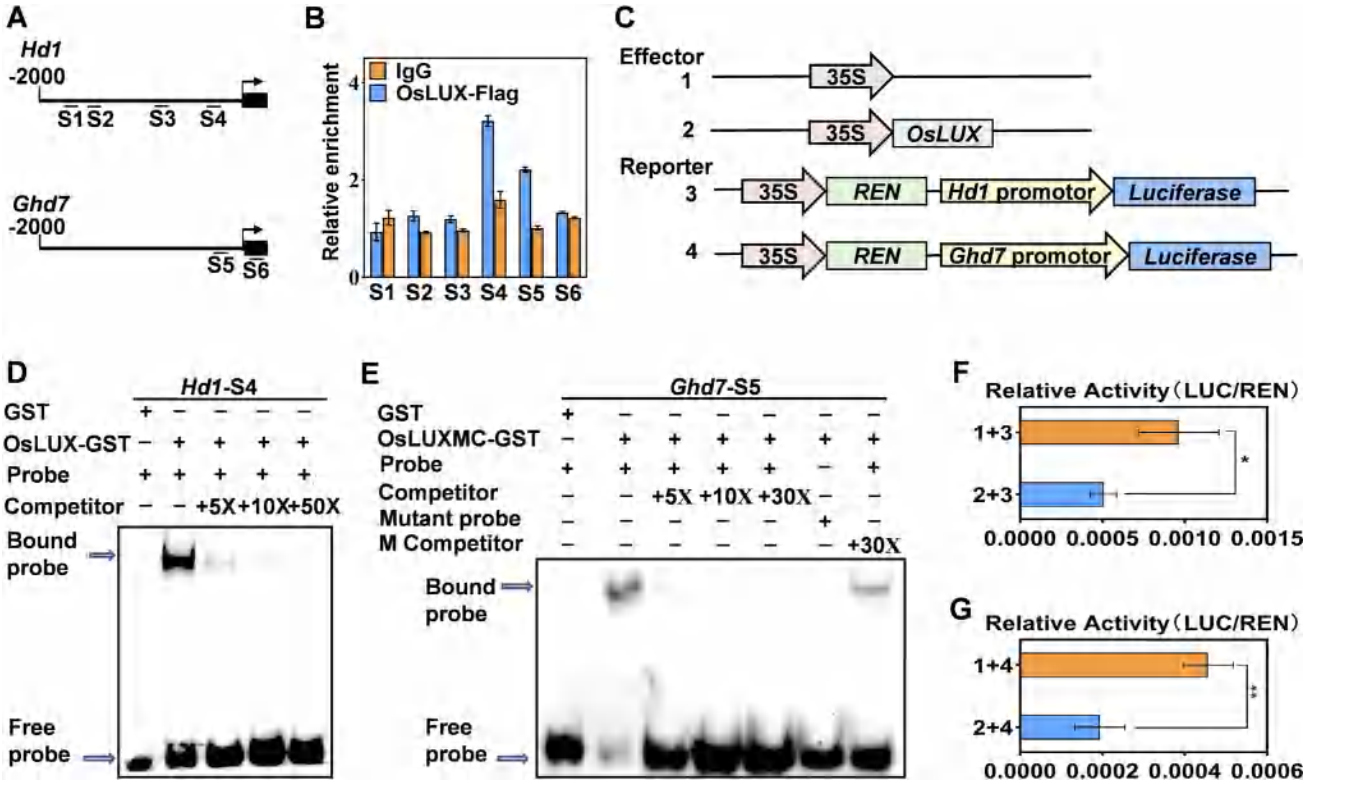

Fig. 6. The OsEC complex suppresses *Hd1* and *Ghd7* expression via binding to the LBS element in their promoter. (A) Diagram of the *Hd1* and *Ghd7* promoter region (2-kb upstream) shows LBS (S1-S6) locations and fragments used for ChIP-qPCR. (B) ChIP-qPCR analysis of *Hd1* and *Ghd7* promoter fragments with 35S::FLAG::OsLUX plants, ChIP was performed with antibodies specific for FLAG and IgG as a control. (C) Diagram of the various constructs used in the transcriptional activity assay. (D and E) EMSA showing the OsLUX protein bound directly to *Hd1* (D) and *Ghd7* (E) promoter, unlabeled probes (five-, Ten-, thirty- and fiftyfold) or mutated probe (thirtyfold) were used in competition assays, the top arrow indicates the shifted band, and bottom arrow indicates the free probe. (F and G) The relative LUC activity values indicate the transcriptional activity. LUC, *firefly* LUC protein, and REN, *Renilla* LUC protein, as a control). The error bars shown mean ± SD, * $P < 0.05$, ** $P < 0.01$.

ferase luminescence was reduced (Fig. S12A-C), showing that the OsELF3-1-OsLUX dimer complex could enhance the suppressive activities of OsLUX to *Hd1*. Moreover, when the p*35S*::*OsELF4s* were further added, we found that the luciferase luminescence was further reduced (Fig. S12A-C), suggesting that the OsELF4s–OsELF3-1–OsLUX complex has the most potent suppression effect on *Hd1*. There is a parallel case that the OsEC complex has a similar suppression effect on *Ghd7* (Fig. S12D-F). Taken together, these results indicate that the OsEC complex directly binds to *Hd1* and *Ghd7* promoters and suppresses their expression.

Discussion

The circadian clock regulates numerous growth and development processes throughout the whole growth cycle of plants. Among the circadian clock-regulated events, one of the most characterized is the internal circadian rhythms, and the external environment functionally works coordinately to regulate photoperiodic flowering. As a core clock regulator, *LUX* has been reported to play multiple roles in the circadian clock and output pathway that regulates plant growth, photoperiodic flowering, freezing tolerance, and defense response [9]. This study revealed that *OsLUX*, which encodes an MYB-like DNA-binding transcription factor, acts as a positive regulator of rice heading through modulating the expression of flowering-time-related genes.

OsLUX plays a critical regulatory role in rice heading

In *Arabidopsis*, *LUX* functions as a flowering repressor, and the mutation in *LUX* exhibits an arrhythmic and early flowering phenotype [6,11,15,39]. In this study, we isolated three mutant alleles of *OsLUX* (*elh1*, *elh2*, and *elh3*), both *elh1* and *elh2* containing a stop-gain mutation showed late heading for more than two months under both NLDs and NSDs. The *elh3* mutant possesses amino acid substitution in the MYB DNA-binding domain, which delayed heading for almost one month compared to Nip under both conditions (Fig. 1A-C; Fig. S4). So, based on our findings, we conclude that the *OsLUX* is indispensable for heading in rice. First, *OsLUX* affects the expression of multiple central clock genes, including *OsPRR37*, *OsPRR73*, *OsPRR1*, *OsPRR59*, *OsPRR95*, and *OsLHY*/*OsCCA1*, which are expressed with peaks at different times of day (Fig. S6). Consistent with LUX repressing the expressions of *Arabidopsis* orthologs of these rice clock genes [6,], these *OsLUX*-regulated clock genes control flowering through the photoperiodic output pathway, such as *OsPRR37* and *OsPRR73* negatively regulate the expression of *Ehd1* to modulate rice heading under LDs [23,24], *OsLHY*/*OsCCA1* possesses dual flowering effect to depend on *OsGI*–*Hd1* pathway under both LDs and SDs [25]. In addition, the highly late heading phenotypes of *oslux* were similar to overexpression of *OsGI*, which up-regulates *Hd1* during the light period and down-regulates *Hd3a* under both SDs and LDs [33].

Unlike in *Arabidopsis*, barley (*Hordeum vulgare*), and *Medicago truncatula*, the mutation in *LUX* homologs causes the photoperiod-insensitive early flowering by elevating the expression of *FT* [39]. In contrast, *oslux* caused an extremely late heading phenotype under both SDs and LDs. This situation is likely to different molecular mechanisms regulating *FT* expression. In *Arabidopsis*, the *CO*-*FT* pathway is the rhythmic output of the biological clock to regulate flowering. *MtLUX* mutation in *Medicago truncatula* caused early flowering due to the up-regulation of the *MtFTa1* but not in a *CO*-like dependent manner [40].

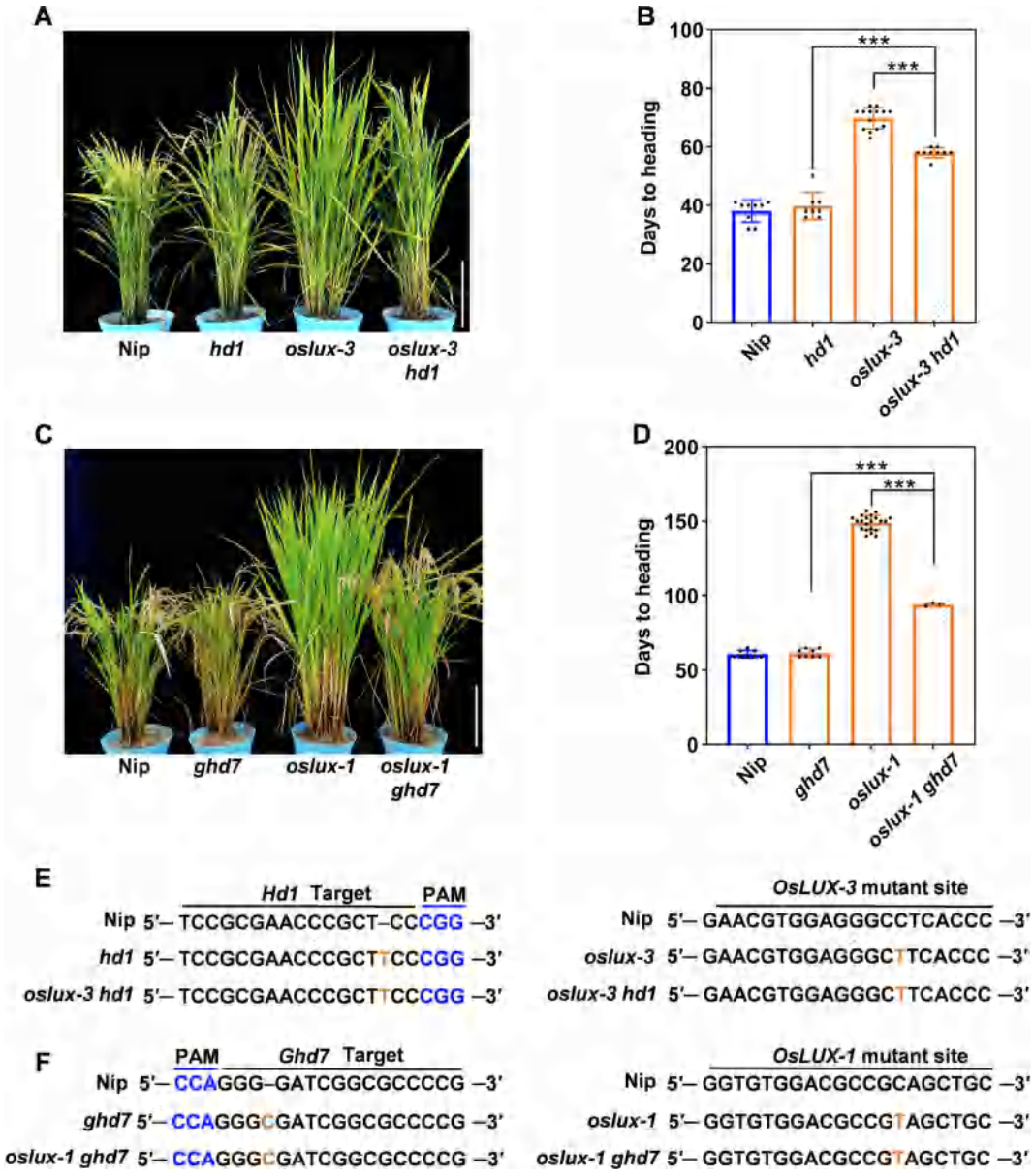

Fig. 7. The phenotype and heading data of the *oslux-3 hd1* and *oslux-1 ghd7* under NSDs in Hainan. (A and B) The phenotype (A) and days to heading (B) of Nip, *hd1*, *oslux-3*, and *oslux-3 hd1* under NSDs. (C and D) The phenotype (C) and days to heading (D) of Nip, *oslux-1*, and *oslux-1* under NSDs. (E and F) The genotype of mutants at *Hd1* and *OsLUX* sites (E) and *Ghd7* and *OsLUX* sites (F). The blue characters indicate the PAM sequence, and the tangerine characters indicate the mutant site. All statistical data represent mean ± SD. *** indicates $P < 0.001$. Bar = 20 cm.

In contrast to *Medicago truncatula*, we found that *OsLUX* regulates the diurnal expression of *Hd1* (a *CO* homolog) in rice. Although the situation is similar to *Arabidopsis CO*, *Hd1* activates *Hd3a* (an *FT* homolog) expression in SDs but suppresses it in LDs [41], indicating the conservative function of *LUX* in flowering-time control but a different regulatory mechanism in rice. In our study, *OsLUX* promotes heading by suppressing the expression of *OsGI* and *Hd1* under the whole photoperiod instead of the light period only under both SDs and LDs. In fact, the *OsLUX-OsGI-Hd1* regulatory module is not enough to thoroughly explain the extreme late-heading phenotype due to the elevated and undetected transcripts of *Ghd7* and *Ehd1* in *elh1*, respectively (Fig. 2C-D, 2 K-L), these results indicate that *OsLUX* modulates rice heading mainly through suppressing the expression of *Hd1* and *Ghd7*. Therefore, we uncover the underlying role of *OsLUX* in the circadian clock and its positive effect on heading in rice.

OsLUX recruits OsELF3-1 and OsELF4s to directly suppress Hd1 and Ghd7 expressions via binding to the LBS in their promoters

Among the EC components, merely LUX possesses a direct DNA binding activity and is responsible for numerous effects such as control flowering, hypocotyl elongation, and defense response through directly binding to the LBS elements alone or with other interaction factors [9,42]. One previous ChIP-seq data reported that the G-box elements are highly enriched at EC binding peaks, indicating that multiple G-box binding transcription factors may co-binding with the EC to regulate the output pathway [37]. In this study, our data showed that OsELF3-1 acts as a bridge to the ternary complex formation (OsELF4s–OsELF3-1–OsLUX) supported by Y2H, pull-down, BiFC, and LCA in vitro and *in vivo* (Fig. 3). In *Arabidopsis*, loss of function in any EC components (*lux*, *elf3*, and *elf4*) results in a similar early flowering phenotype [10]. However,

the mutation in the component of OsEC (*OsLUX*, *OsELF3-1*, and *OsELF4s*) complex in rice showed the various extent of late heading phenotype, particularly in *oslux*, exhibited an extremely late-flowering phenotype, indicating the conservative function but a distinct molecular mechanism for OsEC to regulate rice heading. In *Arabidopsis*, at least 800 LBSs were found in the genome [9], which supports the importance of *LUX* to maintain clock function and regulate multiple output pathways via direct control of multiple downstream target genes. Although one recent study has reported that the OsEC1 (OsELF4a–OsELF3-1–OsLUX) complex was found to coordinately regulate salt tolerance and heading in rice through targeted regulation of *OsGI* [35]. However, it is unknown whether OsLUX directly targeted the regulation of multiple genes located downstream of *OsGI* in the photoperiodic flowering pathway like in *Arabidopsis*. Thus, further research on OsEC-regulated genes could expand our understanding of OsEC's function in regulating heading in rice.

Interestingly, our study found OsLUX directly and explicitly binds to the promoters of *Hd1* and *Ghd7*, supported by EMSA and Chip-qPCR assay (Fig. 6A-E). The LCA assays suggest that the OsLUX suppresses the target genes alone or coordinates with OsELF3-1 and OsELF4s (Fig. 6F-G, Fig. S12A-F). Our data support the pivotal role of *OsLUX* in mediating heading function through direct regulation of expression of key flowering genes in output pathways.

The complete OsEC complex is required for control rice heading

Hd1 and *Ghd7* are the essential genes of two flowering signal pathways with partial crosstalk [43]. Previously, Saito et al. (2012) reported that *OsELF3-1*/*Ef7* modulates rice heading though negatively affecting *Hd1* and *Ghd7*, while the molecular mechanisms are not fully domesticated. Based on our findings and previous research, in this study, the OsELF4s–OsELF3-1–OsLUX–*Hd1*/*Ghd7* regulatory model was proposed to illustrate the flowering mechanism of the OsEC complex (Fig. 8). The OsELF3-1–OsLUX complexes were co-located in the nucleus, whereas the OsLUX showed a dual cytosolic–nuclear localization (Fig. 4A). OsLUX is imported into the nucleus depending on interaction with OsELF3-1, discovered only in the nucleus (Fig. 4A-B). These results suggest that OsELF3-1 retains bound OsLUX in the nucleus and helps OsLUX enter the nucleus to improve its repression functions. Besides, we found that OsELF4s could enhance the interaction between OsLUX and OsELF3-1 (Fig. S10), consistent with the role of ELF4 in increasing nuclear localization of ELF3 in *Arabidopsis* [36]. Collectively, OsELF4s and OsELF3-1 mediate photoperiodic flowering pathways by increasing OsLUX nuclear accumulation to positively affect its function.

Given the dual functional regulation of *Hd1* under distinct photoperiod conditions, we wondered how the OsEC complex mediates rice heading through targeted repression of the expression

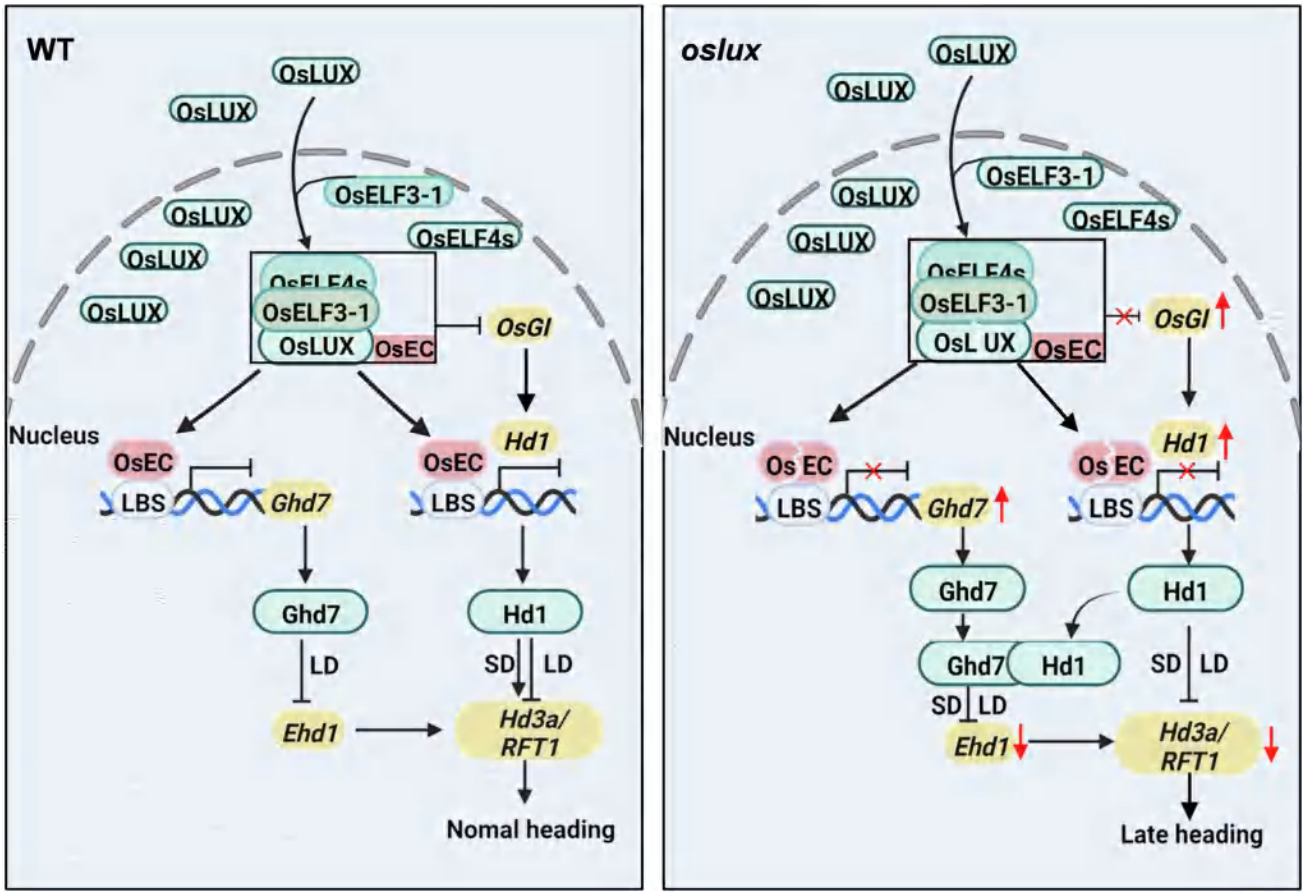

Fig. 8. A working model shows how the OsELF4s-OsELF3-1-OsLUX complex functions to regulate rice heading. In WT, the OsELF3-1 contributes to the translocation of OsLUX to the nucleus. The OsELF4s interact with the heterodimer OsELF3-1-OsLUX to form a trimer complex to suppress the transcription of the vital flowering-time-related genes *Hd1* and *Ghd7* through directly binding to the "LBS" elements of their promoter, hence regulating heading in rice. In *oslux*, defect in OsLUX causes the suppression function of the OsEC complex to be entirely forfeit, which enhances the repression effect of Hd1-Ghd7 on *Ehd1* and *Hd3a* under both LDs and SDs, thus, resulting in a late heading phenotype.

of *Hd1* and *Ghd7* under both LDs and SDs. Recently reported that Ghd7 forms a dimer complex with Hd1 to suppress the expression of *Ehd1* and *Hd3a* during light periods to delay heading under LDs [43]. *Hd1* promotes heading under SDs due to the too low abundance of Ghd7 to generate Hd1-Ghd7 repressive complexes, and a similar pattern was observed in *ghd7* and *dth8* under LDs [17,43–46]. In our study, the phenotype of highly late heading was caused by a functional deficiency in *OsLUX* under both SDs and LDs. The expression levels of *Hd1* and *Ghd7* were significantly up-regulated as well as a similar expression pattern in *elh1* under both SDs and LDs (Fig. 2A-B, K-L), which means more Hd1 and Ghd7 proteins abundance may be accumulated in *elh1* since OLUX released its disincentive effects on *Hd1* and *Ghd7* under both photoperiod conditions. Thus, Hd1-Ghd7 repressive complexes play a predominant role in delays heading in *elh1* under both SDs and LDs. This is why the almost unobservable *Ehd1* and *Hd3a* transcripts in *elh1* under SDs are similar to those under LDs.

Both OsELF3-1 and OsELF4-1 overexpressing plants showed early heading under LDs and late heading under SDs [28,35]. Our finding may explain the dual phenotype resulting from the strength of OsEC target repression, where OsELF4-1 and OsELF3-1 increased the repressive strength of OsEC for targeted regulation of *Hd1* and *Ghd7* in their overexpressing plants (Fig. S12A-F). Therefore, in over-expression plants, the down-regulated *Hd1* and *Ghd7* transcripts further relieved the suppression of *Ehd1* and *Hd3a*, then caused an early heading at LDs. Under SDs, *Hd1* exerts its promotion function in the background of a low level of *Ghd7*, and the low transcription levels of *Hd1* will cause a delayed heading [43–45]. On the contrary, defects in *OsELF4s* and *OsELF3-1* weakened the repressive strength of OsEC to regulate *Hd1* and *Ghd7*. In *oself4s* mutants, OsELF4s failed to improve the interaction between OsLUX and OsELF3-1, while OsLUX-OsELF3-1 dimer still plays a predominant role in target repression. Thus, a minor effect on heading in *oself4s* was observed, which delayed heading slightly late than Nip (Fig. 5C-D; Fig. S11A-D). As for *oself3-1*, the repression strength of OsEC is significantly weakened, maybe due to OsLUX being unable to enter the nucleus completely. Consequently, in this study, the mutation in *OsELF3-1* caused a more significant flowering effect that exhibited 22.1-d and 18.2-d later heading than WT plants under NLDs and NSDs, respectively (Fig. 5A-B). Defect in *OsLUX* resulted in the suppression function of the OsEC complex being entirely forfeit, which utterly released the targeted repression of *Hd1* and *Ghd7*. Thus, an extreme late heading phenotype in the *elh1* results from the nearly invisible level of *Ehd1* and *Hd3a* under both LDs and SDs (Fig. 1A-B, Fig. 2C-F). Together, the complete OsEC complex is required to control rice heading via repress *Hd1* and *Ghd7* expression.

Conclusion

Our findings show that the MYB family transcription factor *OsLUX* is involved in the rice heading by directly regulating rhythm oscillation and core flowering-time-related genes. We uncovered a mechanism by which OsEC (OsELF4s–OsELF3-1–OsLUX) target suppresses the expressions of *Hd1* and *Ghd7* directly to modulate rice photoperiodic flowering. The OsEC–*Hd1*/*Ghd7* regulatory module provides the genetic targets for crop improvement by selecting various combinations of OsEC–*Hd1*/*Ghd7* components, which achieved optimization of photoperiod and planting area.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.08.001.

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