OsLHY regulates photoperiodic flowering through the unique pathways under long-day conditions in rice (Oryza sativa L.)

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Abstract

Flowering time (or heading date in crops) is a critical agronomic trait for rice reproduction and adaptation. The circadian clock is an endogenous oscillator that is involved in controlling photoperiodic flowering. The rice LATE ELONGATED HYPOCOTYL (OsLHY), the core oscillator component of circadian clock, is a homolog of the LHY/CCA1 in Arabidopsis. Here we showed that CRISPR/Cas9-engineered mutations in OsLHY caused late flowering in rice only under long-day (LD) conditions. In the oslhy mutant, the diurnal expression of circadian clock-related genes was seriously affected under both LD and short-day (SD) conditions. Furthermore, the expression of the flowering activators Ehd1, Hd3a and RFT1 was down-regulated and flowering repressors Hd1 and Ghd7 was up-regulated in the oslhy mutant under LD conditions. While the transcripts of flowering-related genes were not dramatically influenced under SD conditions. Dual-luciferase assays showed that OsLHY repressed the transcription of OsGI, Hd1, Ghd7, Hd3a, RFT1 and OsELF3, and activated the transcription of Ehd1. Moreover, the yeast one hybrid assay confirmed that OsLHY directly repressed OsGI, RFT1 and OsELF3 by binding to their promoters, which is consistent with that in Arabidopsis. These results suggested that the OsLHY can promote rice flowering mainly through regulating Hd1 and Ehd1 under LD conditions.

1 Introduction

Rice (*Oryza sativa*) is one of the most important food crops and feeds over half of the world population. Flowering time determines the cropping seasons and regions, and appropriate flowering time will benefit for successful reproduction (Izawa, 2007; Sun et al., 2014). Photoperiodic flowering is the major flowering pathway in rice, which is controlled by both the internal circadian rhythm and the environmental cue, such as day length and light (Shim, Kubota, & Imaizumi, 2017). The regulations of circadian clock and photoperiodic flowering have been widely studied in the model long-day (LD) plant, *Arabidopsis* (Johansson & Staiger, 2015; Shim et al., 2017; Song, Shim, Kinmonth-Schultz, & Imaizumi, 2015). While it is limited study on the connection between circadian clock and photoperiodic flowering in the model short-day (SD) plant, rice.

The *Arabidopsis* CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and its functional paralog LATE ELONGATED HYPOCOTYL (LHY) are the important part of circadian clock oscillator (Schaffer et al., 1998; Wang & Tobin, 1998). CCA1 and LHY are the hub components of multiple interlocked feedback loops, which required to maintain circadian rhythms (Locke et al., 2006; McClung, 2014). CCA1, LHY and PSEUDO-RESPONSE REGULATOR1 (PRR1) comprise the first central negative transcriptional feedback loop. The *CCA1* and *LHY* can be activated at dawn to repress the transcription of evening-expressed *PRR1* by directly binding to its promoter. Whereas *PRR1* peaks at dusk, which in inverse inhibits the expression of *CCA1* and *LHY* (Alabadi et al., 2001; Gendron et al., 2012). Meanwhile, *CCA1* , *LHY* and two PRR family

members *PRR7* and *PRR9* also form a negative feedback loop. CCA1 and LHY repress the expression of *PRR7* and*PRR9* , and then PRR7 and PRR9 along with PRR5 directly suppress the expression of *CCA1* and *LHY* (Farre, Harmer, Harmon, Yanovsky, & Kay, 2005; Kamioka et al., 2016; Nakamichi et al., 2010).

In *Arabidopsis* , *GIGANTEA* (*GI*) acts as a positive regulator of *CONSTANS* (*CO*), which then activates the florigen gene *FLOWERING LOCUS T* (*FT*) under LD conditions (Yano et al., 2000), which form the *Arabidopsis* major photoperiodic flowering pathway. The *Arabidopsis GI -CO -FT* photoperiodic flowering pathway is partially conserved with the *OsGI -Heading date 1*(*Hd1*)-*Heading date 3a* (*Hd3a*)/ *RICE FLOWERING LOCUS T 1* (*RFT1*) pathway in rice. OsGI activtates the expression of *CO* ortholog *Hd1* in the conserved pathway (Hayama, Yokoi, Tamaki, Yano, & Shimamoto, 2003). However, the *Hd1* has a dual function in controlling flowering, which promotes photoperiodic flowering under SD conditions, while inhibits photoperiodic flowering under LD conditions (Yano et al., 2000). There are two florigen genes*Hd3a* and *RFT1* in rice, the *Hd3a* more likely induces flowering under SD, and *RFT1* induces flowering under LD conditions (Kojima et al., 2002; Komiya, Ikegami, Tamaki, Yokoi, & Shimamoto, 2008; Komiya, Yokoi, & Shimamoto, 2009). In addition, the other photoperiodic flowering pathway: *Grain number, plant height, and heading date 7* (*Ghd7*)-*Early heading date 1*(*Ehd1*)-*Hd3a /RFT1* pathway is unique present in rice.*Ehd1* functions as flowering inducer through activating the expression of *Hd3a* and *RFT1* under both LD and SD conditions (Doi et al., 2004). Under LD conditions, the expression of*Ghd7* is enhanced and then *Ghd7* suppresses the expression of *Ehd1* to delay floral transition, suggesting that *Ghd7*acts as a flowering repressor (Xue et al., 2008). Furthermore, Ghd7 could interact with Hd1 to inhibit flowering by repressing *Ehd1* , which indicated that the two pathways are not strictly independent in controlling photoperiodic flowering (Nemoto, Nonoue, Yano, & Izawa, 2016).

In *Arabidopsis* , *CCA1* and *LHY* function in the regulation network of photoperiodic flowering (Mizoguchi et al., 2002; Z. Y. Wang & Tobin, 1998). Loss-of-function of *CCA1* and*LHY* lead to early flowering (Mizoguchi et al., 2002) and constitutive expression of *CCA1* resulted in late flowering in*Arabidopsis* (Wang & Tobin, 1998). CCA1 cooperates or acts in parallel with ELF3 to regulate flowering time (Lu et al., 2012). Moreover, CCA1/LHY also represses the transcription of *GI* and*FT* via directly binding to their promoters, and then suppresses the photoperiodic flowering (Lu et al., 2012; Park, Kwon, Gil, & Park, 2016).

However, there is only single copy of LHY/CCA1 ortholog in rice (Murakami, Tago, Yamashino, & Mizuno, 2007). And it is still unknown how the function of *OsLHY* in photoperiodic flowering in rice. Here, we generated *OsLHY* -defective mutants by CRISPR/Cas9 genome editing system, which exhibited delayed flowering phenotypes under LD, but not SD conditions. The circadian clock was significantly affected in the *oslhy* under both LD and SD conditions, confirming that the photoperiodic flowering was associated with circadian rhythm in high plants (Johansson & Staiger, 2015; Song et al., 2015). As a positive regulator of photoperiodic flowering in rice, OsLHY could suppress the expression of *OsGI* , *Hd1* and *Ghd7* and activate*Ehd1* , but has transcriptional repression effect on *Hd3a*and *RFT1* in tobacco leaves. Furthermore, OsLHY can directly bind to *RFT1* promoter in yeast, similar to that in*Arabidopsis* . While actually, the transcription of *Hd3a* and*RFT1* was significantly reduced in *oslhy* mutants under LD conditions. These results indicated that OsLHY regulated the expression of *Hd3a* and *RFT1* to promote flowering mainly through indirect manners in rice.

2 Materials and methods

2.1 Plant materials and growth conditions

The *oslhy* mutants were generated by the CRISPR/Cas9 system on the Nipponbare (WT) background. Mutants and WT were grown in the field under natural summer conditions (day length >13.5, natural LD). The *oslhy* mutants and WT were also grown in the growth chambers at 30°C under LD photoperiod (14 h light/10 h dark) and SD photoperiod (10 h light/14 h dark), respectively. Heading date was recorded when the first panicle emergence to about 2 cm from the leaf sheath after germination.

2.2 Plasmid Construction and Plant Transformation

Two target sequences were designed in the fifth exon of *OsLHY* gene using the web tool CRISPR-P (*http://cbi.hzau.edu.cn/crispr/*) (Lei et al., 2014). The original CRISPR/Cas9 vector was kindly provided by Prof. Yao-Guang Liu from South China Agricultural University. CRISPR/Cas9 was constructed according to the protocol previously described (Ma et al., 2015). Then *Agrobacterium* -mediated transformation was performed to introduce the binary constructs into EHA105 and rice callus generated from WT. The targets are listed in Supplemental Table 1.

2.3 DNA extraction and mutation type detection

Genomic DNA was extracted from the leaves of rice transgenic plants using the CTAB method (Murray & Thompson, 1980). The DNA containing the targeted sequence regions was amplified and sequenced and relative primers are listed in Supplemental Table 1. The genotypes of targeted mutations were further analyzed by using DSDecode, the web-based tool (http://skl.scau.edu.cn/dsdecode/) (Liu et al., 2015).

2.4 Subcellular localization

The full length coding sequence of *OsLHY* was fused to green fluorescent protein (GFP) in the pM999 vector under the control of the CaMV 35S promoter. Meanwhile, the RPL1 coding sequencing was used to fuse with cyan fluorescent protein (CFP), as *RPL1* can be used as a nuclear marker (Zhang, Yuan, & Zhang, 2012). Then the two plasmids were co-transformed into the rice etiolated stems protoplasts isolated from ten-day-old Zhong Hua11, which was grown in the growth chamber at 28 °C under dark conditions. The method for rice protoplasts transformation was based on the previously study (Bart, Chern, Park, Bartley, & Ronald, 2006). Fluorescence signals were detected by an Olympus FluoView FV1000 confocal microscope.

2.5 RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

For expression pattern analysis of *OsLHY* , various tissues were collected from WT plants under natural LD conditions. For others, the plants were grown under natural LD conditions for 28 days (d), then were separately transferred to growth chambers with LD and SD conditions. After being entrained 14 d, leaves were separately harvested every 4 h during a 24 h period from WT and the *oslhy* mutant under LD and SD conditions. All collected samples with three replicates were frozen in liquid nitrogen and stored at -80 . Total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. A total amount of 1 μg RNA per sample was used for reverse transcription. cDNAs were synthesized using PrimeScript RT reagent Kit with gDNA Eraser following the manufacturer's instructions (Code NO. RR047A Takara, Japan). The qRT-PCR was performed using SYBR Premix Ex Taq II (Code NO. RR820A/B Takara, Japan) with the Roche Real-Time PCR instrument (LightCycler 480). Sequence-specific primers were used for qRT-PCR (Supplemental Table 1) with *ACTIN* as an internal control. The qRT-PCR analysis was performed in three technical replicates for each sample. Relative gene expression levels were calculated using the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2001).

2.6 Luciferase imaging and dual-luciferase assays

The promoters of flowering related genes were inserted into the pGreenII 0800-LUC vector to drive the firefly luciferase (*LUC*) gene that used as the reporter. The pGreenII 0800-LUC vector also contains a renilla luciferase (*REN*) gene driven by CaMV35S that served as positive control. While the effector vector carried the full coding sequence of *OsLHY* under the control of CaMV35S promoter. The 35S::GFP construct was used as a negative control. All constructs were introduced into the *Agrobacterium* strain EHA105-pSoup. The primers used for related vector construction are listed in Supplemental Table 1.

The tobacco (*Nicotiana benthamiana*) plants were grown in the growth chamber at 25 under 16 h light/8 h dark photoperiod. Dual-luciferase assays were performed with approximately four-week-old tobacco leaves. The reporter and effector constructs were mixed in a ratio of 1:10 (v/v) and injected into tobacco leaves via needleless syringes. The negative control and tested pairs were injected into the same leaves but in different positions. At least 48 h after injection, the leaves were sampled for fluorescence signal observation and LUC activity detection. The luciferin (100 μM) was sprayed into the infiltrated tobacco leaves, and then were kept in a dark condition for 5 min before fluorescence observation. Tanon 5200 imaging system was used

to capture the fluorescence signals images. The LUC and REN activities were assayed with dual-luciferase assay reagents (Promega) by using the GloMax 96 microplate luminometer (Promega), according to the manual provided by the manufacturer. The effect of OsLHY on the transcriptional activity of the tested flowering regulators was finally determined by the relative ratio of LUC/REN. At least five biological repeat were measured for each samples.

2.7 Yeast one-hybrid assay (Y1H)

Yeast one-hybrid assay (Y1H) was performed to verify the DNA-protein interaction following the manual of the Matchmaker one-hybrid system (Clontech). The full-length cDNA of *OsLHY* was cloned to fuse with the GAL4 activation domain in the pGAD7-Rec2 vector. The promoters of flowering related genes were amplified to insert into the pHIS2 vector to generate reporter constructs. Subsequently, these reporter plasmids were separately co-transformed with the fusion plasmid pGADT7-OsLHY into the yeast Y187. The co-transformed yeast cells were grown on the SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His media containing 50 mM 3-amino-1,2,4-triazole (3-AT) in a 30incubator for 3-5 d. Relative primers are shown in Supplemental Table 1.

3 Results

3.1 Loss-of-function of *OsLHY* leads to delayed heading date

To investigate the function of OsLHY in photoperiodic flowering in rice, we generated gene editing mutants using CRISPR/Cas9 technology. Totally, nine editing mutants were obtained (Supplemental Figure 1) and they all displayed late heading date phenotype in the growth chamber at 30°C with LD conditions (data not shown). Then in T_1 generation, three independent homozygotes *oslhy-1* , *oslhy-4* and*oslhy-5* were selected for further analyses. These three mutants caused open reading frame shift and resulted in premature stop codons. As expected, the *oslhy* mutants exhibited delayed flowering phenotype when grown under natural LD conditions. The heading date was delayed by approximately 14 d, 12 d and 15 d related to WT, respectively (Figures 1B and 1C). However, no significantly difference was observed between *oslhy* mutants and WT under SD conditions (Supplemental Figure 2A and 2B). The*oslhy* mutants also showed more tiller numbers, shorter panicle length, less thousand grain weight and higher seed setting rate than that of WT (Supplemental Table 2), which was consistent to previous studies (Chaudhury, Dalal, & Sheoran, 2019; Wang et al., 2020). The expression of *OsLHY* was detected by RT-PCR and showed declined expression in *oslhy* mutants when compared to WT (Figure 1D).

3.2 *OsLHY* expression patterns and subcellular localization

The qRT-PCR was performed to understand the expression patterns of *OsLHY* in various tissues from root, stem, leaf blade, glum, pistil, anther and eight different panicle developmental stages. The results showed that *OsLHY* was expressed in all investigated tissues, with the relatively highest mRNA transcription level in leaf (Figure 2A), in accordance with the fact that *OsLHY* could involve in the regulation of photoperiodic flowering. Since *OsLHY* encodes a putative MYB transcription factor, the rice protoplast transient expression was performed to investigate the subcellular localization. The fluorescence signal of OsLHY-GFP overlapped with the fluorescence signal of nuclear mark (RPL1) (Figure 2B), indicating that OsLHY was localized in the nucleus, which was consistent with the LHY/CCA1 in *Arabidopsis* (Liu, Covington, Fankhauser, Chory, & Wagner, 2001).

3.3 Expression rhythms of circadian clock components is altered in the *oslhy* mutant

As a circadian clock gene, the expression rhythm of *OsLHY* was examined in the *oslhy* mutant and WT under both LD and SD conditions. The *OsLHY* gene remained obvious rhythmic expression in the *oslhy* mutant just like that in WT, which peaked at dawn (ZT0) and reached its trough around dusk (ZT12) under both LD and SD conditions (Figure 3A and 3B). While it was reduced on the daytime and increased on the nighttime in the *oslhy* mutant when compared to WT (Figure 3A and 3B). The other seven circadian clock genes (*OsPRR1* , *OsPRR37* , *OsPRR73* , *OsPRR59* ,*OsPRR95* , *OsGI* and *OsELF3*) also kept clear rhythmic expression in the *oslhy* mutant and WT under both LD and SD conditions, but the expression

was severely influenced and the peak time of these genes was almost shifted earlier under both LD and SD conditions (Figure 3).*OsPRR73*and *OsPRR37* were strongly repressed throughout the daytime in the*oslhy* mutant under LD conditions (Figure 3C and 3E), but significantly increased in the morning (ZT0 and ZT4) under SD conditions (Figure 3D and 3F). *OsPRR95* and *OsPRR59* were also significantly increased in the morning (ZT0 and ZT4) in the *oslhy* mutant under both LD and SD conditions (Figure 3G-3J). The expression of *OsPRR1* and *OsGI* were significantly increased in the early points of daytime in the*oslhy* mutant (ZT4 and ZT8 under LD conditions, ZT4 for*OsPRR1* and ZT0, ZT4 and ZT8 for *OsGI* under SD conditions) (Figure 3K-3N). In the *oslhy* mutant, the transcription of*OsELF3* was significantly higher than that of WT at the beginning of the day under LD conditions, while *OsELF3* was significantly down-regulated from ZT8 to ZT20. Under SD conditions, the transcription of *OsELF3* was increased at ZT0 and ZT12 (Figure 3O and 3P). Additionally, in the *oslhy* mutant, the expression peaks of*OsPRR73* , *OsPRR37* , *OsPRR59* , *OsPRR95* were advanced by approximately 4 h or 8 h, leading to their expression peak time at ZT4 under both LD and SD conditions (Figure 3C-J). Under LD condition, the peak time of *OsPRR1* and *OsGI* shifted approximately 4 h early and peaked at ZT8 in the *oslhy* mutant (Figure 3K and 3M). Under SD condition, the peak time of *OsPRR1*was not changed and also peaked at ZT8 in the *oslhy* mutant (Figure 3L). Yet the peak time of *OsGI* was advanced by approximately 4 h and peaked at ZT4 in the *oslhy* mutant (Figure 3N). These expression peak time shifts were consistent with circadian clock genes highly expressed at early daytime (Figure 3). Taken together, these results indicated that the core circadian clock gene*OsLHY* regulated the rhythmic expression of all tested circadian clock genes under both LD and SD conditions.

3.4 The expression of flowering-related genes dramatically influenced in *oslhy* mutants

As OsGI and OsELF3 are also important regulators on photoperiodic flowering pathways in rice (Hayama et al., 2003; Izawa et al., 2011; Yang, Peng, Chen, Li, & Wu, 2013) and the expression of *OsGI* and*OsELF3* was obvious affected in the *oslhy* mutant. Therefore, the expression of flowering-related genes (*Hd1* ,*Ehd1* , *Ghd7* , *Hd3a* and *RFT1*) was further investigated (Figure 4). As expected, the expression of *Hd1* and*Ghd7* was dramatically increased, while *Ehd1* , *Hd3a*and *RFT1* was dramatically decreased in *oslhy* mutations under LD conditions (Figure 4A). Under SD conditions, the expression of*Ghd7* was increased, and *Hd1* , *Ehd1* , *Hd3a* and*RFT1* was not significantly affected in *oslhy* mutations (Figure 4B).

3.5 OsLHY affects the transcriptional activity of flowering related genes

To further explore the effects of OsLHY on the transcription of flowering related genes, the dual-luciferase detections were performed in tobacco leaves. The signals of LUC triggered by the *OsGI* , *Hd1* , *Ghd7* , *Hd3a,RFT1* and *OsELF3* promoters in tobacco leaves were weakened by introducing the OsLHY protein (Figure 5A-C, 5E-F and Supplemental Figure 3). While the signal of LUC triggered by *Ehd1* promoter in tobacco leaves was strongly increased when the OsLHY was co-expressed (Figure 5D). These results indicated that OsLHY has transcriptional suppression activity on *OsGI* , *Hd1* , *Ghd7* ,*Hd3a* and *RFT1* promoters, while has transcriptional activation activity on the *Ehd1* promoter.

3.6 OsLHY physically binds to the promoters of flowering related genes

The clock genes *LHY* and *CCA1* regulate multiple genes mainly though directly binding to their promoters in *Arabidopsis*(Alabadi et al., 2001; Lu et al., 2012; Park et al., 2016). The EE expanded sequences were predicted to be bound by OsLHY using web tools JASPAR (http://jaspar.genereg.net/) (Supplemental Figure 4A) (Fornes et al., 2020). The 2 kb promoter regions of seven flowering-related genes (*OsELF3* , *OsGI* ,*Hd1, Ghd7* , *Ehd1, Hd3a* and *RFT1*) were scanned using web tools MEME (http://meme-suite.org/tools/fimo) (Bailey et al., 2009; Grant, Bailey, & Noble, 2011), showed that their promoters contain the EE expanded binding sites (Supplemental Figure 4B and Supplementary Table 3). Therefore, it was speculated that*OsLHY* could regulate flowering-related genes through directly interacting with their promoters.

Y1H was performed to confirm their interactions, and found that the co-transformants of pGADT7-OsLHY along with pHIS2-P*OsGI* , pHIS2-P*OsELF3* and pHIS2-P*RFT1* could grow on the selection medium SD/-Leu/-Trp/-His in the presence of 50 mM 3-AT, similar to the positive control (pHis2-P*53* and pGADT7-53),

whereas the co-transformants of pGADT7-OsLHY along with pHIS2-P*Hd1* , pHIS2-P*Ghd7* , pHIS2-P*Ehd1* and pHIS2-P*Hd3a* were similar to the negative control (pHis2-P*53* and pGADT7- OsLHY), failed to grow on the selection medium (Figure 6). These results indicated that OsLHY can physically bind to *OsGI* , *OsELF3*and *RFT1* promoters in *vivo* . While it cannot directly bind to the promoters of *Hd1* , *Ghd7* , *Ehd1* and

Hd3a.

4 Discussion

4.1 OsLHY has essential effect on the circadian clock

As one of the central clock oscillator components, loss-of-function of*OsLHY* obviously affected the rhythmic expression pattern of circadian clock-related genes. In *Arabidopsis* , *LHY* and*CCA1* defective mutants showed shortened circadian periods (Green & Tobin, 1999; Mizoguchi et al., 2002; Niwa et al., 2007). In the*oslhy* mutant, the expression peaks of *OsPRR73* ,*OsPRR37* , *OsPRR95* , *OsPRR59* , *OsPRR1* and*OsGI* were shifted early (Figure 3), suggested that *OsLHY* defective mutants could also result in shortened circadian periods.*HY* and *CCA1* can inhibit the transcription of almost all circadian clock-related genes in the morning (Nagel & Kay, 2012; Sanchez & Kay, 2016). The expression of *OsPRR73* and*OsPRR37* was significantly reduced under LD conditions, but increased under SD conditions in *oslhy* mutants. While*OsPRR95* , *OsPRR59* , *OsPRR1* , *OsGI* and*OsELF3* was increased in *oslhy* mutants under LD conditions (Figure 3), which indicated that OsLHY may suppress these genes in early time of the daytime. The expression of *OsPRR1* was increased in*oslhy* mutants (Figure 3K and 3L), because *OsLHY* (or*OsCCA1*) could repress its expression by directly binding to its promoter (Wang et al., 2020). In addition, *OsLHY* was highly expressed in the *osprr1* mutant (Wang et al., 2020), which may confirm the speculation that *OsLHY* and *OsPRR1* are components of the central negative feedback loop in rice (Yang et al., 2013). Overexpression of *OsPRR37* suppressed the expression of*OsLHY* under LD conditions (Liu et al., 2018), indicated that reciprocally regulation between *OsLHY* and *OsPRR37* is complicated. In *Arabidopsis,* CCA1 suppresses GI and ELF3 by interacting directly with their promoters, which add additional connections between the circadian clock and the flowering pathways (Lu et al., 2012). The expression of *OsGI* was increased at some early daytime points (Figure 3M and 3N), and the tobacco transient expression and Y1H showed that OsLHY can directly repress the transcription of*OsGI* and *OsELF3* (Figure 5A, Supplemental Figure 4 and Figure 6). These data revealed that the additional connections among circadian clock and the flowering pathways are conserved between rice and *Arabidopsis* .

4.2 OsLHY promotes flowering by regulating *Hd1*- and*Ehd1*-mediated pathways under LD conditions

Overexpression of *OsLHY* in *Arabidopsis* displayed the late flowering independent of the day length, which is consistent with the phenotype of overexpression of *LHY* or *CCA1* (Murakami et al., 2007; Wang & Tobin, 1998). Dysfunction of *LHY* and*CCA1* in *Arabidopsis* exhibited an early flowering phenotype under both LD and SD conditions (Mizoguchi et al., 2002). Whereas, late flowering was observed in *oslhy* mutants specially under LD conditions (Figure 1B). The mutation in *OsCCA1* leads to the delayed flowering phenotype (Wang et al., 2020), suggested that the role of LHY/CCA1 is not fully conserved between *Arabidopsis* and rice in controlling of photoperiodic flowering. This distinct role may result from partially conserved photoperiodic pathway between*Arabidopsis* and rice. In *Arabidopsis* , CO induces flowering through activating the expression of *FT* under LD conditions (Kobayashi, Kaya, Goto, Iwabuchi, & Araki, 1999). CCA1 and LHY could directly repress the transcription of *GI* to inhibit flowering (Lu et al., 2012; Park et al., 2016). And CCA1 also directly represses*FT* via binding to its promoter (Park et al., 2016). Additionally,*ELF3* is directly suppressed by CCA1, in contrast, *CCA1* is indirectly activated by ELF3 through PRR9 (Lu et al., 2012). Under LD conditions, ELF3 functions mainly upstream of *CCA1* to delay flowering (Lu et al., 2012). There are two major photoperiodic flowering pathways in rice: the *Hd1* - and *Ehd1* -mediated pathways (Komiya et al., 2009; Tsuji, Taoka, & Shimamoto, 2011). The*Hd1* -mediated pathway is conserved with the CO-mediated pathway in*Arabidopsis* (Yano et al., 2000). OsGI promotes the expression of*Hd1* , then

Hd1 induce the transcription of *Hd3a* and*RFT1* to promote flowering under SD conditions, but represses it by down-regulated of *Hd3a* and *RFT1* under LD conditions (Figure 7) (Kojima et al., 2002; Yano et al., 2000). Besides, *Hd1*also participates in the *Ehd1* -mediated pathway (Doi et al., 2004). It can interact with Ghd7 to form a complex, which then represses the transcription of *Ehd1* through binding to its promoter under LD conditions (Nemoto et al., 2016). In the *Ehd1* -mediated pathway, Ghd7 and Ehd1 are two unique flowering regulators which act as the flowering suppressor and flowering activator, respectively (Doi et al., 2004; Xue et al., 2008). OsELF3 functions as a flowering activator upstream of *OsGI* and *Ghd7* under LD conditions and it may promote the expression of *OsLHY* by directly repress *OsPRRs*(Figure 7) (Yang et al., 2013). The dual luciferase assays showed that OsLHY has transcriptional suppression effect on *OsGI* , *Hd1* ,*Ghd7* , *Hd3a* , *RFT1* and *OsELF3* , while it has transcriptional activation effect on *Ehd1* (Figure 5 and Supplemental Figure 3). The Y1H showed that OsLHY also could bind to promoters of *OsGI* , *RFT1* and *OsELF3* (Figure 6), suggested that though OsLHY directly represses the expression of*RFT1,* it could increase the expression of *Hd3a* and*RFT1* via both *Hd1* - and *Ehd1* -mediated pathways under LD conditions. As OsLHY could mitigate the inhibition effect of Hd1 on*Hd3a* and *RFT1* by OsGI-dependent or OsGI-independent suppression. Furthermore, OsLHY could repress the transcription of *Ghd7* and promote the transcription of *Ehd1* , which will result in increased expression of *Hd3a* and *RFT1* (Figure 7).

Although OsLHY repressed the transcription of *OsGI* ,*OsELF3* , *Hd3a* and *RFT1* in tobacco leaves (Figure 5A, E, F and Supplemental Figure 3), the expression of *OsGI* ,*Hd3a* , *RFT1* and *OsELF3* was all significantly reduced under LD conditions in the *oslhy* mutant related to WT (Figure 3M, 3O and Figure 4A). However, the expression of *Hd1* and *Ghd7*(Figure 4A) was increased and *Ehd1* was decreased in *oslhy*mutants under LD conditions, which was consistent with the results that OsLHY repressed the transcription of *Hd1* and *Ghd7* and activated the transcription *Ehd1* in tobacco leaves (Figure 5B-D). It indicated that *OsLHY* may mainly regulate the expression of*Hd3a* and *RFT1* through *Hd1* - and*Ehd1* -medicated pathways in rice.

The expression pattern of *OsGI* under SD is similar with that under LD conditions, and the expression of *Ghd7* was increased in*oslhy* mutants under SD conditions. However, the expression of*Hd1* , *Ehd1* , *Hd3a* and *RFT1* was not severely impaired in *oslhy* mutant under SD conditions. It may explain why*oslhy* mutants showed no obviously phenotype on flowering time under SD conditions. Taken together, it allowed us to deduce that OsLHY acts as the positive flowering activator by modulating the transcription of *Hd1* and *Ehd1* under LD conditions.

4.3 The function of LHY/CCA1 on flowering is distinct in LD and SD plants.

In *Arabidopsis* , *LHY* and *CCA1* inhibit flowering under both LD and SD conditions (Mizoguchi et al., 2002; Wang & Tobin, 1998). The quadruple mutant of *LHY/CCA1* -LIKE orthologs*GmLCLa1 a2 b1 b2* lead to delayed flowering in soybean under LD conditions (Wang et al., 2019). While *oslhy* mutants exhibited late flowering in rice only under LD conditions, which similar as observed in soybean but different from that in *Arabidopsis* . The possible reason may be that the soybean and rice are both SD plants, yet the *Arabidopsis* is a LD plant. It allowed us to hypothesize that whether the *LHY/CCA1* genes activate or inhibit flowering varies by species and is related to the photoperiod response of the species. Notably, it is not unique phenomenon. The function of PRRs and OsGI on controlling flowering are also associated with the photoperiod response in diverse species (Bendix, Marshall, & Harmon, 2015). In general, GI activates flowering in LD plants and suppresses flowering in SD plants (Bendix et al., 2015). While LHY/CCA1 may inhibits flowering in LD plants and activates flowering in SD plants.

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Conflict of interest

The authors declared no conflict of interest in the authorship and publication of this document.

Author contributions

JHX and CL designed research; CL, XJL, YY, EKY and JHX performed research; CL, YY, MSA, and RFT conducted the field work; CL, ZL, MHD and JHX analyzed data; CL and JHX wrote and edited the manuscript.

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