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RESEARCH REPORT

OsFLZ2 interacts with OsMADS51 to fine-tune rice flowering time

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ABSTRACT

Flowering time is an important agronomic trait affecting crop yield. FCS-LIKE ZINC FINGER (FLZ) proteins are plant-specific regulatory proteins that are involved in multiple biological processes. However, their roles in plant flowering time control have not been clarified. Here, we report that OsFLZ2 is a negative regulator of rice flowering time. OsFLZ2 delays flowering by repressing the expression of key floral integrator genes. Biochemical assays showed OsFLZ2 physically interacts with OsMADS51, a flowering activator under short-day (SD) conditions. Both *OsFLZ2* and *OsMADS51* are highly expressed in rice leaves before floral transition under natural SD conditions, and their proteins are colocalized in the nucleus. Co-expression of OsFLZ2 can destabilize OsMADS51 and weaken its transcriptional activation of the downstream target gene *Early heading date 1* (*Ehd1*). Taken together, these results indicate that OsFLZ2 can interfere with the function of OsMADS51 to fine-tune rice flowering time.

KEY WORDS: Flowering time, FLZ, OsMADS51, Ehd1, Rice

INTRODUCTION

Rice is one of the most important crops worldwide. Appropriate flowering time (also known as heading date) is crucial for rice regional adaptation and optimal yield determination (Hayama et al., 2003; Izawa, 2007; Tsuji et al., 2011; Zhao et al., 2015). Precocious flowering shortens vegetative development available for carbon assimilation and causes yield reduction. On the other hand, delayed flowering could lead to reduction of seed setting in late growing season and trigger yield loss. Thus, identifying flowering time regulatory genes and exploring the underlying molecular mechanisms is crucial for rice genetic improvement (Zhou et al., 2021).

Flowering time in rice is strongly affected by exogenous factors, among which photoperiod is a key factor. As a typical facultative short-day (SD) plant, rice harbors at least two independent photoperiodic flowering pathways (Zhou et al., 2021). The first one is the *Heading date 1* (*Hd1*; *SE1*) pathway (Yano et al., 2000), which is conserved both in rice (*OsGI-Hd1-Hd3a*) and in *Arabidopsis* (*GI-CO-FT*) (Tsuji et al., 2011; Shrestha et al., 2014). *Arabidopsis* GI-CO-FT signaling flow is active under long-day (LD) conditions, whereas rice OsGI-Hd1-Hd3a is active under SD conditions to promote flowering (Tsuji et al., 2011; Shrestha et al., 2014). Another photoperiodic flowering pathway in rice is the *Early heading date 1* (*Ehd1*; *EF1*) pathway (Doi et al., 2004). *Ehd1* was originally cloned from a major flowering time quantitative trait locus and encodes a B-type response regulator (Doi et al., 2004) that can promote flowering by inducing expression of the florigenic genes *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T1* (*RFT1*), regardless of Hd1 function or day-length conditions (Doi et al., 2004; Nemoto et al., 2016). The precise regulation of *Ehd1* expression is crucial for the appropriate heading date and several proteins involved in this process have been identified (Lee et al., 2004; Kim et al., 2007; Xue et al., 2008; Wei et al., 2010; Yan et al., 2011; Nemoto et al., 2016; Zhou et al., 2021). For example, OsMADS51, an ortholog of *Arabidopsis* FLOWERING LOCUS C (FLC) (Ruelens et al., 2013), functions as an upstream transcriptional activator of *Ehd1* (Kim et al., 2007). The transcript level of *Ehd1* is increased in *OsMADS51* overexpression plants, but decreased in *osmads51* mutants. Further study has revealed that OsMADS51 acts downstream of *OsGI* and can transmit a promotional signal from *OsGI* to *Ehd1* under SD conditions, thus promoting flowering (Kim et al., 2007). The outline of OsMADS51-Ehd1-Hd3a flowering pathway has been established, but the detail regulatory mechanisms remain to be further characterized.

FCS-LIKE ZINC FINGER (FLZ) proteins are plant-specific regulatory proteins containing a conserved FLZ domain (also known as Domain of Unknown Function 581) (Jamsheer and Laxmi, 2014). Studies in *Arabidopsis thaliana*, maize (*Zea mays*) and wheat (*Triticum aestivum*) have demonstrated that FLZ proteins are implicated in the plant stress response (Jamsheer and Laxmi, 2015; Jamsheer et al., 2019; Chen et al., 2021; Qin et al., 2021). For example, ectopic expression of *TaFLZ2D* confers better tolerance of transgenic plants to salt stress (Qin et al., 2021). Recently, we systematically identified 29 OsFLZ genes in the rice genome and demonstrated that *OsFLZ18* negatively regulates rice submergence tolerance by interacting with Snf1-related protein kinase 1A (SnRK1A) and inhibiting the transcriptional activation activity of SnRK1A in modulating the expression of its target gene *αAmy3* (Ma et al., 2021). However, the roles of FLZ proteins in rice growth and development, especially in flowering transition, have not yet been well clarified. Here, we reported that *OsFLZ2* (LOC_Os01g41010) negatively regulates rice flowering time.

RESULTS AND DISCUSSION

OsFLZ2 is a negative regulator of flowering time

Most OsFLZ genes are ubiquitously but differentially expressed in various tissues or at different developmental stages, implying their important roles in rice growth and development (Ma et al., 2021).

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To investigate whether and how OsFLZ genes regulate rice growth and development, we used the CRISPR/Cas9 gene editing system to generate OsFLZ mutants and observed the phenotypical difference from wild-type Nipponbare (Nip) plants. Among the obtained mutants, we found two *OsFLZ2* Crispr lines (Crispr-4 and Crispr-11, with, respectively, 43- and 1-nucleotide deletions, both resulting in a predicted truncated OsFLZ2 protein, Fig. 1A) that flowered earlier than Nip plants (Fig. 1C,D and Fig. S1). Under natural LD conditions (NLD, from mid-April to July, Guangzhou, China), *OsFLZ2* Crispr lines flowered ∼4 days earlier than Nip. Under natural SD conditions (NSD, from mid-July to October, Guangzhou, China), they flowered ∼3 days earlier than Nip (Fig. 1C,D and Fig. S1). These observations indicated that OsFLZ2 negatively regulates rice heading date. To further confirm this notion, transgenic rice plants overexpressing *OsFLZ2* (OE-2 and OE-7, Fig. 1B) were generated in the Nip background. As expected, the OE transgenic plants flowered 4-5 days later than Nip under NLD conditions and 6-7 days later under NSD conditions (Fig. 1C,D and Fig. S1). These results indicate that *OsFLZ2* is a negative regulator of rice flowering time.

OsFLZ2 affects the expression of floral integrator genes

To elucidate the roles of *OsFLZ2* in regulating heading date, we examined the expression levels of four key flowering-related integrator genes (*Hd1*, *Ehd1*, *Hd3a* and *RFT1*) using quantitative real-time PCR assay (qRT-PCR, Fig. 2). Diurnal gene expression patterns were investigated using 14- and 30-day-old Nip and *OsFLZ2* transgenic plants grown under SD (10 h light) and LD (14 h light) conditions, respectively. Under SD conditions, the transcript levels of *Hd1*, *Ehd1* and *Hd3a* were greatly increased in the Crispr-4 line, particularly at the time points of peak expression in Nip, whereas they were considerably decreased in the OE-2 line when compared with those in Nip (Fig. 2A). In addition, the expression of *RFT1* was comparable between Crispr-4 and Nip, whereas it was lower in OE-2 lines (Fig. 2A). Under LD conditions, all these four genes generally showed increased expression in the Crispr-4 line but decreased expression in the OE-2 line at the peak time points, when compared with those in Nip (Fig. 2B). These results indicate that OsFLZ2 can downregulate the expression of key flowering-promoting genes to delay rice heading date.

OsFLZ2 interacts with OsMADS51 transcription factor

To date, no evidence showed FLZ proteins can function as transcription factors to directly modulate gene expression. To explore how *OsFLZ2* regulates flowering-time gene expression, we explored OsFLZ2-interacting proteins using immunoprecipitation followed by a mass spectrometry (IP-MS) assay. In this assay, we obtained 353 potential OsFLZ2-binding proteins, including the previously described proteins SnRK1A and SnRK1B (Table S1; Ma et al., 2021). Interestingly, one high-scored peptide of OsMADS51

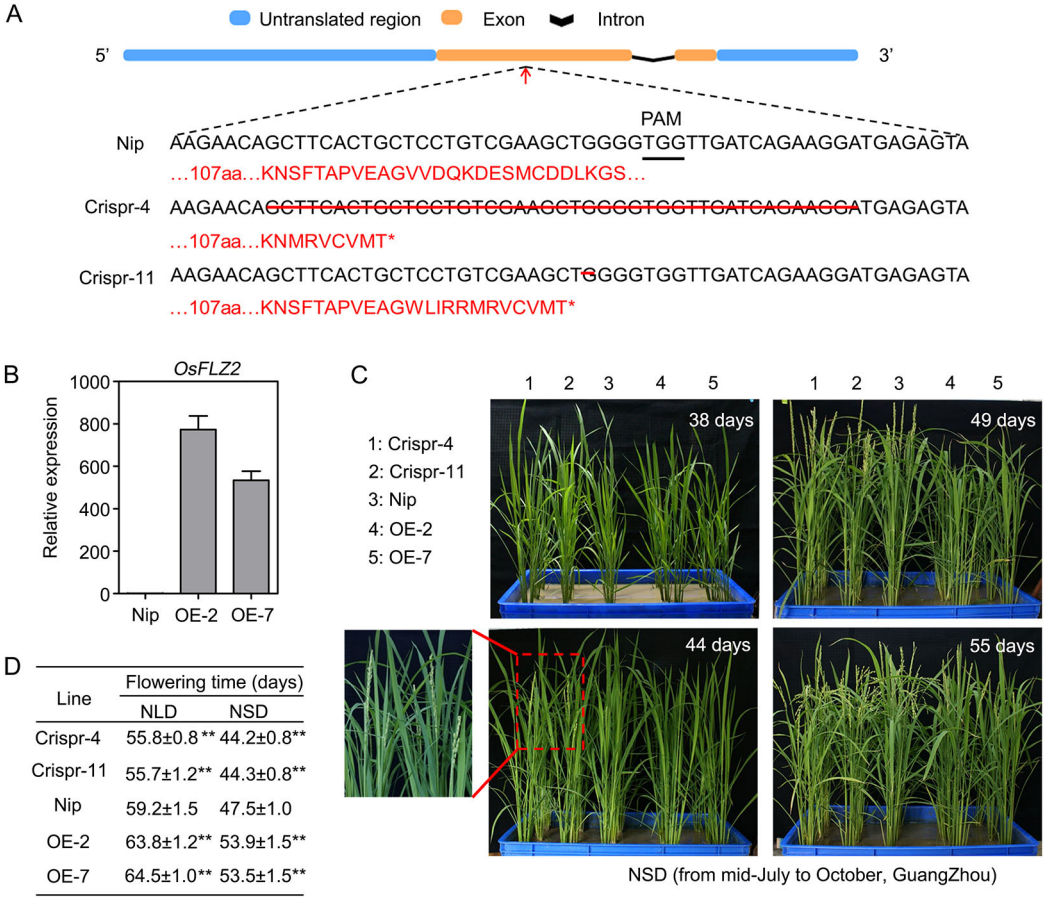

Fig. 1. *OsFLZ2* negatively regulates rice flowering time. (A) Schematic of two *OsFLZ2* CRISPR/Cas9 mutants. The strikethrough indicates base deletion. Red letters indicate amino acids. Asterisks indicate stop codons. PAM, protospacer adjacent motif. (B) qRT-PCR analysis of *OsFLZ2* expression in Nip and two *OsFLZ2*-OE lines. *Ubiquitin* was used as an internal control. Data are mean±s.d. (n=3). (C) Flowering phenotypes of Nip and *OsFLZ2* transgenic plants under natural short-day (NSD, from mid-July to October, Guangzhou, China) conditions. (D) Flowering time of the indicated lines under NSD and natural long-day conditions (NLD, from mid-April to July, Guangzhou, China) conditions. Data are means±s.d. (n=6). **P<0.01, unpaired two-tailed Student's t-test.

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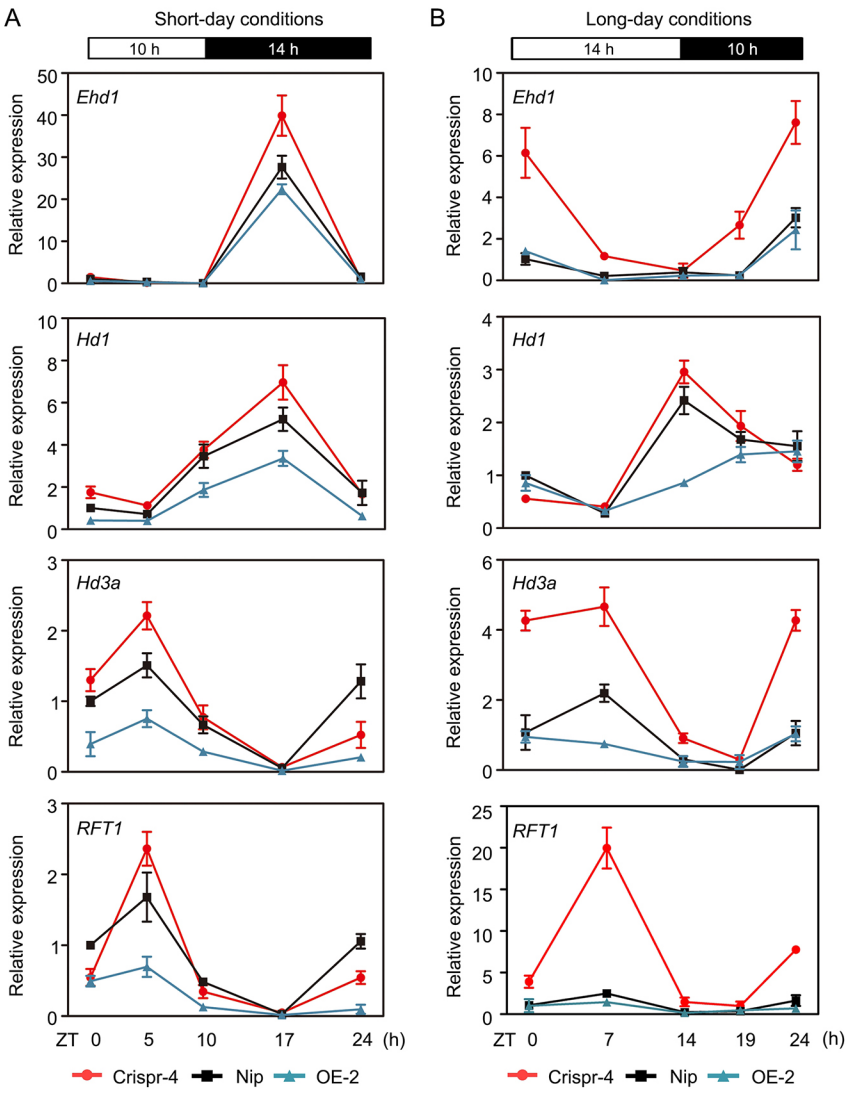

Fig. 2. Diurnal expression of flowering integrator genes in Nipponbare and *OsFLZ2* transgenic plants under short-day and long-day conditions. (A,B) Penultimate leaves were collected at the indicated time points from 14- and 30-day-old rice plants grown under short-day (A; 10 h light) and long-day (B; 14 h light) conditions, respectively. The expression levels are relative to *Ubiquitin* and the values of Nipponbare are set as '1.0' at ZT0. Data are means±s.d. (n=3). The white bars and black bars at the top represent the light and dark periods, respectively. ZT, Zeitgeber time.

was identified (Fig. 3A and Table S1). OsMADS51 is a type I MADS transcription factor that functions as a floral activator by promoting the expression of *Ehd1*, *Hd3a* and *RFT1* (Kim et al., 2007). Therefore, we proposed that OsFLZ2 might interact with OsMADS51 to regulate flowering gene expression. To test this, we first confirmed the OsFLZ2-OsMADS51 interaction in detail (Fig. 3B-D). In glutathione S-transferase (GST) pull-down assay, His-OsMADS51 protein could be effectively pulled down by GST-OsFLZ2, but not by GST alone (Fig. 3B), demonstrating a direct interaction between OsFLZ2 and OsMADS51. The interaction was further corroborated by a co-immunoprecipitation (co-IP) assay. Tobacco (*Nicotiana benthamiana*) leaves expressing OsFLZ2-GFP/OsMADS51-6HA and GFP/OsMADS51-6HA were harvested to isolate total proteins followed by an immunoprecipitation assay. Western blotting analysis showed that OsMADS51-6HA could be successfully co-precipitated by OsFLZ2-GFP, but not by GFP (Fig. 3C). This interaction was also assessed by firefly luciferase complementation imaging (LCI) assay. As shown in Fig. 3D, the co-expression of FLZ2-nLUC with cLUC-MADS51 led to strong LUC signals in tobacco leaves, whereas no obvious signal was observed from the co-expression of the negative controls, further supporting the association of OsFLZ2 and OsMADS51 in plant cells. Other members of MADS family, such as OsMADS8, OsMADS15 and OsMADS50, have also been reported to modulate rice heading date (Kang et al., 1997; Lee et al., 2004; Bian et al., 2011; Lu et al., 2012), so we checked the interaction of OsFLZ2 with them. The obtained LCI results showed that OsFLZ2 can interact with OsMADS50, but not with OsMADS8 and OsMADS14 (Fig. S2).

OsFLZ2 exhibits a declining and diurnal rhythmic expression in leaves following rice growth

OsMADS51 functions early in the first 2 months of growth in rice leaves to activate flowering under SD conditions (Kim et al., 2007). To examine the expression profile of *OsFLZ2* in leaves following rice growth, we harvested penultimate leaves from Nip plants every week from seedling (3 weeks old) to heading stage (9 weeks old) under NSD conditions to perform a qRT-PCR assay. Consistent with the previous results (Kim et al., 2007), OsMADS51 showed declining expression after rice growth (Fig. 4A). Similarly, *OsFLZ2* also displayed its highest expression in leaves at the seedling stage (3 weeks old), followed by a gradually decreased expression to tillering stage (5 weeks old) and remained low until heading stage (9 weeks old) (Fig. 4A). The flowering time of Nip is roughly 7 weeks under NSD conditions in Guangzhou and the flowering transition occurs before 5 weeks. Thus, the expression of *OsFLZ2* was higher

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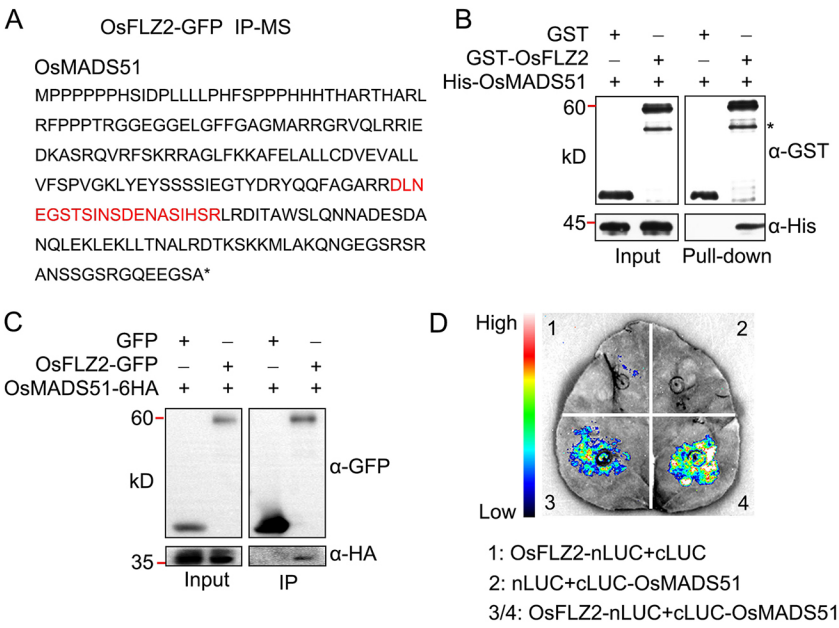

Fig. 3. OsFLZ2 physically interacts with OsMADS51. (A) The identified peptide of OsMADS51 in the immunoprecipitation-mass spectrometry assay. Red amino acids indicate identified peptide sequence. (B) GST pull-down assay showing the direct interaction between OsFLZ2 and OsMADS51. Asterisk indicates the non-specific bands. (C) An *in vivo* co-immunoprecipitation assay reveals that OsFLZ2 associates with OsMADS51. (D) The LCI assay shows that OsFLZ2 interacts with OsMADS51 in the leaves of *N. benthamiana*.

before the flowering transition, indicating that *OsFLZ2* functions in flowering determination at the early stage. We then assayed the diurnal expression of *OsFLZ2*. To achieve this, we harvested the penultimate leaves over a 48 h time course from 14-day-old Nip plants under SD conditions (Hori et al., 2013) to measure the transcript level of *OsFLZ2*. The qRT-PCR results showed that *OsFLZ2* has an obvious diurnal rhythmic expression pattern, showing relatively low expression during light periods, high expression at night and a peak at midnight (Fig. S3). Collectively, these data indicate that *OsFLZ2* expression overlapped that of OsMADS51 after rice growth and had an obvious diurnal rhythm.

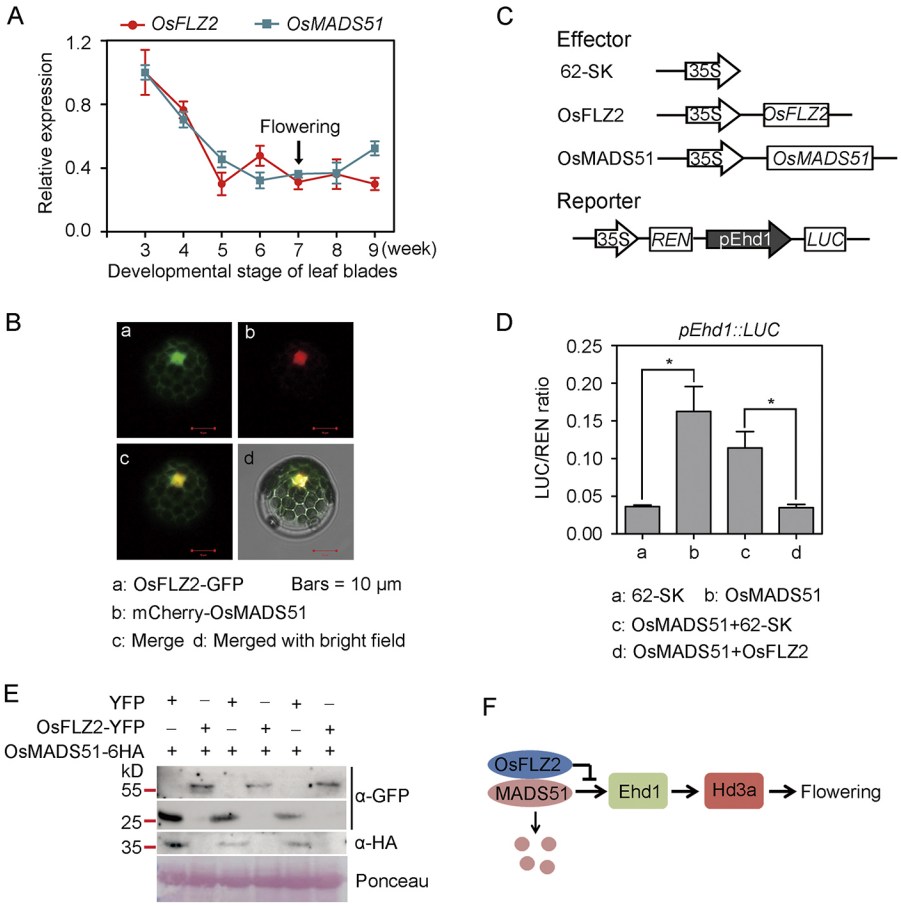

Fig. 4. The effects of OsFLZ2 on the transcriptional activation of *Ehd1* by OsMADS51. (A) qRT-PCR analysis of *OsFLZ2* and *OsMADS51* expression in rice leaves during different growth stages under natural short-day (NSD, from mid-July to October, Guangzhou, China) conditions. The leaf blades were harvested at 6:00 P.M. from 3- to 9-week-old Nipponbare plants. The *Ubiquitin* gene was used as an internal control. The expression level was set as 1.0 for 3-week-old plants. Data are mean±s.d. (n=3). (B) Colocalization of OsFLZ2-GFP and mCherry-OsMADS51 in *Arabidopsis* protoplasts. (C) Schematic diagram of plasmid constructs used in the dual-luciferase assay. (D) Dual-luciferase assay showing the effects of OsFLZ2 on the transcriptional activation of *Ehd1* by OsMADS51. Data are mean±s.d. (n=3), *P<0.05, unpaired two-tailed Student's t-test. (E) Western blotting showing the effect of OsFLZ2 on the protein levels of OsMADS51. (F) A proposed model for the function of the OsFLZ2/OsMADS51 complex in rice heading date regulation (flowering).

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OsFLZ2 destabilizes OsMADS51 and weakens its transcriptional activation of *Ehd1*

To dissect the functional connection between OsFLZ2 and OsMADS51 in the regulation of flowering gene expression, a colocalization analysis was performed (Fig. 4B). The result showed that the fluorescence signal of OsFLZ2-GFP overlapped that of mCherry-OsMADS51 in the nucleus (Fig. 4B), in agreement with their roles in modulating transcription. OsMADS51 serves as a transcriptional activator of *Ehd1* (Kim et al., 2007), whereas *OsFLZ2* represses *Ehd1* expression (Fig. 2); thus, we hypothesized that OsFLZ2 might be able to influence the transcriptional activation of *Ehd1* by OsMADS51. To test this possibility, a dual-luciferase analysis was performed using *Ehd1* promoter to drive a *LUC* reporter gene (Fig. 4C). In line with the previous report (Kim et al., 2007), the expression of the *pEhd1*::*LUC* reporter gene was upregulated about fourfold by addition of OsMADS51 (Fig. 4D), suggesting activation of *Ehd1* by OsMADS51. However, co-expression OsFLZ2 (62-SK-OsFLZ2) but not the negative control (62-SK empty vector) with OsMADS51 could significantly downregulate the expression of the *pEhd1*::*LUC* reporter gene (Fig. 4D), demonstrating that OsFLZ2 can weaken the transcriptional activation of *Ehd1* by OsMADS51. To clarify how OsFLZ2 involves in this process, we checked the protein levels of OsMADS51 with or without OsFLZ2 co-expression. The data showed that co-expression of OsFLZ2 decreased the protein accumulation of OsMADS51 dramatically (Fig. 4E), indicating that OsFLZ2 can destabilize OsMADS51.

Based on these results, we propose a molecular model regarding the function of OsFLZ2 in rice flowering time control (Fig. 4F). OsFLZ2 interacts with OsMADS51 in the nucleus to destabilize and attenuate the transcriptional activation of *Ehd1* by OsMADS51, in order to decrease the expression of the downstream florigenic genes *Hd3a* and *RFT1*, thus delaying heading (Fig. 4F). Of note, OsMADS51 is a photoperiod-sensitive flowering activator; its overexpressors exhibits an obvious early flowering only under SD conditions (Kim et al., 2007). *OsFLZ2* functions similarly in flowering transition under SD and LD conditions, whereas the transgenic plants exhibited less photoperiodic sensitivity (Fig. 1 and Fig. S1).

OsFLZ2 has a declining expression pattern in leaves following rice growth under SD conditions, which is highly similar to the action of *OsMADS51* (Fig. 4A, Kim et al., 2007), meeting the requirement for their interaction *in vivo*. Additionally, OsFLZ2 colocalizes with OsMADS51 in the nucleus (Fig. 4B) and can attenuate the transcriptional activation activity of OsMADS51 to *Ehd1* (Fig. 4C,D), in strong agreement with the repression of *Ehd1* by OsFLZ2 (Fig. 2). Interestingly, co-expression of OsFLZ2 can decrease the accumulation of OsMADS51 protein (Fig. 4E), indicating that OsFLZ2 can destabilize OsMADS51. In *Arabidopsis*, FLZ proteins serve as scaffolding proteins and can broadly interact with protein kinases, transcription factors and other types of regulatory proteins (Nietzsche et al., 2016). Therefore, it is possible that OsFLZ2 can mediate the interaction of OsMADS51 with its regulatory proteins, such as E3 ubiquitin ligases, to promote its turnover. Beyond OsMADS51, OsFLZ2 also interacts with OsMADS50 (Fig. S2), which exerts a similar function in regulating flowering by promoting *Ehd1* expression (Lee et al., 2004; Bian et al., 2011). It will be interesting to test whether OsFLZ2 also enables the interference functions of OsMADS50 in the same manner.

The transcription of *Hd1* is obviously upregulated in *OsFLZ2* Crispr line under both SD and LD conditions (Fig. 2), indicating that OsFLZ2 also affects *Hd1*-mediated flowering pathway. *Hd1* is a major photoperiod sensitivity flowering regulator in rice that induces flowering under SD conditions and delays it under LD conditions (Yano et al., 2000; Kojima et al., 2002; Hayama et al., 2003; Ishikawa et al., 2011; Nemoto et al., 2016). Previous studies have shown that OsMADS50 and OsMADS51 mainly function under SD conditions to promote flowering, and the expression of *Hd1* is not changed in their mutants or in overexpression plants (Lee et al., 2004; Kim et al., 2007), indicating that *OsFLZ2* regulates *Hd1* expression independently on these two transcription factors.

Collectively, our current study functionally characterizes OsFLZ2 as a novel flowering regulator in rice. We also reveal a hitherto unknown function of FLZ proteins in regulating plant growth, and highlight the functional importance and versatility of FLZ proteins. These findings will broaden our understanding of the roles of FLZ proteins and provide a new gene resource for the regulation of rice flowering.

MATERIALS AND METHODS

Plant materials and growth conditions

The Nipponbare (Nip) was used for rice transformation. For phenotypic observation, *OsFLZ2*-OE, *OsFLZ2*-Crispr and Nip plants were grown at the Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou (23.13°N, 113.27°E), China, where the normal early growing season with natural long-day (NLD) conditions is from mid-April to July and the late growing season with natural short-day (NSD) conditions is from mid-July to October. The heading date was recorded as the day when the first panicle emerged from the leaf sheath. For diurnal expression analysis, *OsFLZ2*-OE, *OsFLZ2*-Crispr and Nip plants were grown in climate chambers under short-day (SD) conditions (10 h light, 28°C/14 h darkness, 25°C) or long-day (LD) conditions (14 h light, 28°C/10 h darkness, 25°C). The penultimate leaves of 14-day-old plants in SD conditions or 30-day-old plants in LD conditions (Hori et al., 2013) were collected at the indicated Zeitgeber time (at the points of light on, light off and the mid-point) for RNA isolation.

Plasmid construction and rice transformation

The full-length coding sequence of *OsFLZ2* was PCR amplified and cloned into the pCAMBIA1300-GFP (Ma et al., 2021) to generate *pUBQ10:OsFLZ2-GFP* construct to express *OsFLZ2*. To generate *OsFLZ2* knockout plants, a 20 bp gene-specific spacer sequence (5′-TGCTCCTGTCG-AAGCTGGGG-3′) of *OsFLZ2* was inserted into the sgRNA/Cas9 construct (Ma et al., 2015). Primers are listed in Table S2. The resulting vectors were confirmed by sequencing and transformed into the *Agrobacterium tumefaciens* strain EHA105, and then introduced into Nip. T_0 transgenic lines of CRISPR/cas9 were analyzed by sequencing to confirm the gene editing. Homozygous *OsFLZ2*-OE (OE-2 and OE-7) and *OsFLZ2*-Crispr (Crispr-4 and Crispr-11) transgenic lines were used for phenotypic observation.

RNA extraction and qRT–PCR analyses

Total RNA was extracted from the collected samples using the Hipure plant RNA Mini Kit (Magen) according to the manufacturer's instructions. The first strand of cDNA was synthesized from 1 µg total RNA using the Primescript RT reagent Kit (Takara) following the manufacturer's instructions. qRT-PCR was performed in a 96-well plate using SYBR Premix ExTaqTM (Takara) on a CFX Connect real-time PCR detection system (Bio-Rad). The following PCR program was used: 95°C for 2 min, 50 cycles of 95°C for 10 s and 60°C for 15 s, followed by a melting-curve program. The rice *Ubiquitin* (*UBQ*) gene was used as an internal control. Relative expression levels of genes were calculated using the $2^{-\triangle\triangle^{CT}}$ method (Livak and Schmittgen, 2001). Primers used for qRT-PCR are listed in Table S2.

Immunoprecipitation-mass spectrometry assay

Leaves of 3-week-old *OsFLZ2-GFP* (OE-2) plants were collected and ground in liquid nitrogen and then homogenized in ice-cold immunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl,

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5 mM $MgCl_2$, 10% glycerol, 0.2% NP-40, 5 mm DTT, 0.1 mM PMSF and proteinase inhibitor cocktail (Roche)]. The suspensions were centrifuged twice at 16,000 ***g*** for 10 min at 4°C to remove debris. The supernatant was incubated with GFP-Trap_MA (gtma-20, ChromoTek) at 4°C for 2 h and then the beads were washed five times with ice-cold immunoprecipitation buffer. After washing, the beads were re-suspended in 30 µl 1×SDS sample loading buffer and boiled at 95°C for 10 min. Finally, all the samples were loaded in 8% stacking gel and ∼1 cm-length gel from the loading pore was cut after running for 15 min at 100 V. The proteins were digested in-gel with trypsin (Guangzhou Huijun Biosciences). Mass spectrometry measurements were performed on a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). The resulting spectra were searched against the RGAP database (http://rice.uga.edu/).

GST pull-down assays

For pull-down assays, the coding sequence of *OsFLZ2* was PCR amplified and cloned into the vector pGEX4T-3, while the coding sequence of OsMADS51 was PCR amplified using KOD polymerase (Toyobo) and cloned into the vector pRSETA-His-SUMO (Ma et al., 2021). Primers are listed in Table S2. The resulting plasmids were separately transformed into *E. coli* BL21 and the proteins were induced with 0.1 mM IPTG. For GST pull-down assay, equal amounts of GST or GST-OsFLZ2 proteins were mixed with His-OsMADS51 at 4°C for overnight in 1 ml binding buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA and 1% NP-40). The beads were then washed three times with binding buffer and boiled at 100°C for 10 min. The elution was subjected to immunoblotting analysis using anti-His (HT501,TransGen; 1:6000) and anti-GST (HT601, TransGen; 1:6000) antibodies.

Co-immunoprecipitation

The full-length coding sequence of *OsFLZ2* was inserted into the pCAMBIA13000-GFP (Ma et al., 2021) vector, while the coding sequence of *OsMADS51* was inserted into pGreen-35S-6HA vector (Qian et al., 2021) to express OsFLZ2-GFP and OsMADS51-6HA, respectively. Primers are listed in Table S2. The resulting plasmids were transformed into *Agrobacterium tumefaciens* (strain GV3101) and then infiltrated into 5-week-old *N. benthamiana* leaves. The total proteins were extracted from leaves expressing OsFLZ2-GFP/OsMADS51-6HA or GFP/OsMADS51-6HA constructs with protein extraction buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 10% glycerol and 1% NP-40] containing 1×protease inhibitor cocktail. An anti-GFP antibody was added to the protein extracts for immunoprecipitation for 3 h, then protein A magnetic beads (36403ES03, Yeasen) were added and incubated for another 2 h. After washing three times with the protein extraction buffer, the co-immunoprecipitated products were separated by SDS-PAGE gel and detected with anti-GFP (HT801,TransGen; 1:5000) and anti-HA (ab9110, Abcam; 1:5000) antibodies.

Firefly luciferase complementation imaging assay

The full-length coding sequence of *OsFLZ2* was PCR amplified and cloned into the pCAMBIA-nLUC (Chen et al., 2008) to generate OsFLZ2-nLUC vector. The full-length *OsMADS8/15/50/51* were PCR amplified and separately cloned into the pCAMBIA-cLUC (Chen et al., 2008) to generate cLUC-OsMADS vectors. Primers are listed in Table S2. These plasmids were transformed into *Agrobacterium tumefaciens* GV3101. *A. tumefaciens* containing OsFLZ2-nLUC, *A. tumefaciens* containing cLUC-OsMADS and *A. tumefaciens* containing P19 were co-injected into fully expanded leaves of 5-week-old *N. Benthamian* plants at a ratio of 2:2:1. After infiltration for 3 days, the infiltrated regions were injected with 1 mM of luciferin (BioVision). The luminescence activity was captured with a chemiluminescent imaging system (Tanon).

Colocalization analysis

The full-length coding sequence of *OsFLZ2* was inserted into pCAMBIA13000-GFP vector to fuse with *GFP*. The full-length coding sequence of *OsMADS51* was inserted into pCAMBIA13000-mCherry (Liu et al., 2021) vector to fuse with *mCherry*. Primers are listed in Table S2. The resulting plasmids were co-transfected into *Arabidopsis* protoplasts following a previously described method (Yang et al., 2018). After incubation in darkness for 16 h, the fluorescence signal of OsFLZ2-GFP and mCherry-OsMADS51 was observed and pictures taken with a confocal fluorescence microscope (LSM 710, Zeiss).

Dual-luciferase reporter assay

The dual-luciferase reporter assay was performed following our previously established method (Yang et al., 2018; Ma et al., 2021). The coding sequence of *OsFLZ2* and *OsMADS51* were separately cloned into the pGreenII 62-SK vector (Ma et al., 2021) to generate the effector vectors. The *Ehd1* promoter fragment (2 kb upstream of the start codon) was amplified and cloned into the pGreenII 0800-LUC (Ma et al., 2021) to generate the reporter vector. The indicated plasmids (10 µg for each) were transfected into *Arabidopsis* protoplasts. After incubation in darkness for 16 h, protoplasts were harvested to extract the total proteins for luciferase activity detection using a Dual-Luciferase Reporter Assay System (E1910, Promega) in accordance with the manufacturer's instructions. The relative luciferase activity was calculated as the ratio of Firefly luciferase activity to Renilla luciferase activity. The experiments were carried out in three independent replicates. Primers used in this assay are listed in Table S2.

Transient expression assay and western blotting

To check the effect of OsFLZ2 on the protein stability of OsMADS51, the coding sequence of *OsFLZ2* was cloned into pSAT6-YFP-N1 (Yang et al., 2018) to generate the OsFLZ2-YFP vector. Primers are listed in Table S2. OsMADS51-6HA (10 µg) was co-transfected into *Arabidopsis* protoplasts with 10 µg YFP or OsFLZ2-YFP. After incubation in darkness for 16 h, protoplasts were harvested to extract the total protein with extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1% NP-40, 5 mm DTT, 0.1 mM PMSF and proteinase inhibitor cocktail (Roche)]. The protein samples were separated by SDS-PAGE gel and detected with anti-GFP (HT801, TransGen; 1:5000) and anti-HA (ab9110, Abcam; 1:5000) antibodies. Ponceau S (ab146313, Abcam) staining of the nitrocellulose membrane indicates the protein loading.

Chemicals and statistical analysis

All chemicals were purchased from Sangon Biotech unless stated otherwise. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test in Microsoft Excel. Statistical difference is shown as $*P \leq 0.05$ and $**P \leq 0.01$. The *n* values represent the number of independent biological replicates. Graphs were produced using GraphPad Prism 5 software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.M., B.L.; Methodology: Y.M., J.D., J.Z., B.L.; Formal analysis: Y.M., W.Y., L.C., W.W., J.W.; Investigation: Y.M., W.L., L.Z., J.C., T.Y., S.Z.; Resources: Y.M.; Writing - original draft: Y.M., J.Z.; Writing - review & editing: B.L.; Supervision: J.Z., B.L.; Project administration: J.Z., B.L.; Funding acquisition: Y.M., J.Z., B.L.

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