OsMFT1 increases spikelets per panicle and delays heading date in rice by suppressing Ehd1, FZP and SEPALLATA-like genes

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Received 17 February 2018; Editorial decision 12 June 2018; Accepted 13 June 2018

Editor: Dabing Zhang, Shanghai Jiao Tong University, China

Abstract

Heading date and panicle architecture are important agronomic traits in rice. Here, we identified a gene MOTHER OF FT AND TFL1 (OsMFT1) that regulates rice heading and panicle architecture. Overexpressing OsMFT1 delayed heading date by over 7 d and greatly increased spikelets per panicle and the number of branches. In contrast, OsMFT1 knockout mutants had an advanced heading date and reduced spikelets per panicle. Overexpression of OsMFT1 significantly suppressed Ehd1 expression, and Ghd7 up-regulated OsMFT1 expression. Double mutants showed that OsMFT1 acted downstream of Ghd7. In addition, transcription factor OsLFL1 was verified to directly bind to the promoter of OsMFT1 via an RY motif and activate the expression of OsMFT1 in vivo and in vitro. RNA-seq and RNA in situ hybridization analysis confirmed that OsMFT1 repressed expression of FZP and five SEPALLATA-like genes, indicating that the transition from branch meristem to spikelet meristem was delayed and thus more panicle branches were produced. Therefore, OsMFT1 is a suppressor of flowering acting downstream of Ghd7 and upstream of Ehd1, and a positive regulator of panicle architecture.

Keywords: Branch meristem, Ghd7, heading date, OsLFL1, OsMFT1, panicle architecture, spikelet meristem.

Introduction

Heading date (flowering time) in rice (Oryza sativa) is crucial for plants to adapt to the growing environment and for the improvement of yield potential. It is determined by the interaction of endogenous signals and environmental factors. Florigen is a key endogenous signal that is synthesized in the leaves and moves to the shoot apex to induce flowering. Many environmental factors could induce or suppress florigen expression. Photoperiod (day length) is the most important environmental factor affecting flowering time (Song et al., 2015). Rice is a typical short-day (SD) plant, one whose flowering is promoted by short daylength. Many genes have been identified as involved in the photoperiod-mediated flowering pathway. Heading date 3a (Hd3a) and RICE FLOWERING LOCUS T1 (RFT1), which are homologous to Arabidopsis thaliana FLOWERING LOCUS T (FT), are florigen genes of rice (Kojima et al., 2002; Tanaki et al., 2007; Komiya et al., 2008; Komiya et al., 2009). In rice, there are two pathways determining floral induction. One is the Heading date 1
(Hd1–Hd3a pathway, which is conserved with the Arabidopsis CONSTANS (CO)–FT pathway. The other is a unique rice pathway, the Ghd7–Ehd1–Hd3a/RFT1 pathway (Song et al., 2015). Hd1, homologous to Arabidopsis CO, promotes Hd3a expression under SD conditions and suppresses Hd3a under long-day (LD) conditions (Yano et al., 2000). *Early heading date 1* (Ehd1) activates Hd3a and RFT1 expression independent of Hd1 in both LD and SD conditions (Doi et al., 2004). *Grain number, plant height, and heading date 7* (Ghd7) suppresses Ehd1 expression. Ghd7 expression is sensitive to photoperiod. Under LD conditions, Ghd7 expression is induced and thus Ehd1 and Hd3a expression is suppressed. Under SD conditions, Ghd7 has low expression and the suppression of Ehd1 is relieved, which allows Ehd1 to induce Hd3a expression (Xue et al., 2008). In rice, a number of flowering genes are found to function by directly or indirectly regulating Ehd1, and thus Ehd1 acts as a floral integrator (Song et al., 2015). A recent discovery showed that Ghd7 interacts with Hdl to delay heading, which indicates these two pathways are not independent in regulating heading date (Nemoto et al., 2016; Zhang et al., 2017).

The rice inflorescence has a branch structure and is usually referred to as a panicle. A rice panicle consists of a main stem, often referred to as the rachis, several primary branches, one or more secondary branches on the primary branch, and occasionally tertiary branches on the secondary branch (Xing and Zhang, 2010). After the transition from the vegetative phase to the reproductive phase, the shoot apical meristem is converted into the inflorescence meristem, and then the inflorescence meristem produces branch meristems. Each branch meristem can continue to produce new branch meristems or transform into a spikelet meristem, and then the spikelet meristem is converted to a floral meristem. In rice, each spikelet meristem generates one flower. Therefore, spikelet meristem identity determines the termination of branch meristem activity. Currently, many genes have been identified as being responsible for the initiation, maintenance, and activity of these meristems (Tanaka et al., 2013; Zhang and Yuan, 2014). A major yield quantitative trait locus (QTL), *GRAIN NUMBER 1a* (GN1a), encodes a cytokinin oxidase, OsCKX2. High levels of cytokinin caused by loss-of-function of OsCKX2 increase branch meristem activity, which is responsible for the increased branch and spikelet number (Ashikari et al., 2005). Rice *FRIZZY PANICLE (FZP)* regulates the transition of branch meristems to spikelet meristems and has a crucial role in establishing the spikelet meristem identity. *fzp* mutants produce branches but fail to form normal spikelets, and spikelets are replaced by branches in mutants carrying severe *fzp* alleles (Chujo et al., 2003; Komatsu et al., 2003; Bai et al., 2016). Another rice yield gene, *TAWAWA1* (TAW1), also regulates spikelet number through suppression of the transition from branch meristems to spikelet meristems. TAW1 promotes branch meristem activity and suppresses the phase change to spikelet meristem identity through positively regulating the *SVP* family MADS-box genes, leading to prolonged branch formation (Yoshida et al., 2013). Thus, promotion of inflorescence meristem or branch meristem activity and appropriate delay of spikelet meristem identity formation could help to increase spikelet number.

Rice *MOTHER OF FT AND TFL1* (MFT) belongs to the family of phosphatidylethanolamine-binding proteins (PEBPs). The PEBP family is a family of evolutionarily conserved genes widely present in eukaryotes (Karlgren et al., 2011). In higher plants, the PEBP gene family consists of three main homologous subfamilies, *FT*-like, *TERMINAL FLOWER1* (TFL1)-like and MFT-like genes (Chardon and Damerval, 2005). As the name suggests, the MFT-like subfamily is a homolog of *FT* and TFL1 and is thought of as the evolutionary ancestor to them (Hedman et al., 2009). In Arabidopsis, there are six PEBP family genes: two *FT*-like genes (*FT* and *TSF*), three TFL1-like genes (*TFL1, BFT*, and *ATC*) and one MFT-like gene (*MFT*) (Danielevskaya et al., 2008). Both FT and TFL1 are key regulators of floral transition but have antagonistic roles. FT has been shown to be florigen and induces flowering while TFL1 has been identified as a flowering suppressor (Alvarez et al., 1992; Kardaiskiy et al., 1999; Hanzawa et al., 2005). In addition to repressing flowering, TFL1 plays a crucial role in determining inflorescence architecture. In Arabidopsis, a main shoot apical meristem produces either indeterminate flowerers or indeterminate lateral axes after floral transition. TFL1 prevents the meristems from assuming the floral identity and accounts for indeterminate growth of the inflorescence shoot. Thus, *FT1* transgenic plants exhibited an extended vegetative phase and branched inflorescence while loss-of-function of TFL1 produced terminal flowers at the shoot apex (Alvarez et al., 1992; Benlloch et al., 2007; Liu et al., 2013). *ARABIDOPSIS THALIANA CENTRORADIALIS* (ATC) and *BROTHER OF FT AND TFL1* (BFT) inhibit flowering similarly to TFL1 (Huang et al., 2012; Yoo et al., 2010), and *TSF* promotes flowering similarly to *FT* (Yamaguchi et al., 2005). As a gene homologous to both *FT* and TFL1, MFT seems to have no major effect on flowering. Overexpression of *MFT* led to slightly early flowering while loss of *MFT* function did not exhibit an obvious phenotype in flowering (Yoo et al., 2004). Later studies showed MFT is involved in the regulation of seed germination via ABA and GA signaling pathways. Loss-of-function of *MFT* led to hypersensitivity to ABA in seed germination. MFT is directly bound by *ABA-INSENSITIVE 3* (*ABI3*) and *ABI5* on the promoter. MFT is suppressed and promoted by *ABI3* and *ABI5*, respectively. In addition, DELLA proteins, the major repressors of GA signaling, could directly bind to the MFT promoter and promote its expression. On the other hand, MFT exerts a negative feedback regulation of ABA signaling by directly repressing *ABI5* (Xi et al., 2010). Besides Arabidopsis, there are several MFT homologs reported to regulate seed germination in other species. In wheat, *TaMFT* is a repressor of seed germination and co-localizes with a seed dormancy QTL (Nakamura et al., 2011). Through ectopic overexpression in Arabidopsis, a Soyben homolog of MFT (*GmMFT*) negatively regulates seed germination, and strawberry homolog of MFT (*FvMFT*) regulates germination via participating in GA and ABA signaling (Li et al., 2014; Hu et al., 2016).

In rice, 19 PEBP genes were identified based on genome-wide analysis, of which there were 13 *FT*-like genes, four *TFL1*-like genes, and two MFT-like genes (Chardon and Damerval, 2005; Danilevskaya et al., 2008). Among them, the most well-studied homolog of *FT* is *Hd3a*. *Hd3a* is a mobile flowering
signal that moves from leaf to shoot apical meristem where it interacts with 14–3–3 protein and OsFD1 to form a flower-specific activation complex, which is essential for the activation of the inflorescence meristem identity gene (Tamaki et al., 2007; Taoka et al., 2011). The four TFL1 homologs in rice are named RCN1, RCN2, RCN3, and RCN4. Overexpression of RCN1, RCN2, and RCN3 exhibited branched dense panicle architecture and delayed heading date, while knocking down of all RCNs produced reduced branches and small panicles (Nakagawa et al., 2002; Zhang et al., 2005; Liu et al., 2013). So far, two MFT homologs in rice, OsMFT1 and OsMFT2, have not been identified yet. A previous study proposed that OsMFT1 was positively regulated by Ghd7 in the flowering pathway through an expression QTL (eQTL)-guided function-related co-expression analysis (Wang et al., 2014). It is very likely that OsMFT1 regulates heading date and panicle architecture. Here, we confirmed that OsMFT1 acts downstream of Ghd7 and elucidated its mechanism in controlling heading and panicle architecture by identification of its upstream and downstream genes.

Materials and methods

Plant material and growth condition

The japonica rice variety Zhonghua 11 (ZH11) was used as the wild type and recipient for genetic transformation. Ghd7-related material, including NIL(mh7), NIL(ps7), OX-Ghd729111, and Ami-Ghd7, were from previous studies (Xue et al., 2008; Weng et al., 2014); the ghd7 mutant had an SNP mutation resulting in a premature stop codon in the ZH11 background. For measurement of the agronomic traits, rice plants grown at Wuhan were under natural LD conditions, whereas plants grown at Hainan were under SD conditions. Germinated seeds were sown in the seed beds and 1-month-old seedlings were transplanted to the fields with 10 plants in a row. The heading date was the day when the first panicle of the plant emerged. Plants in the middle of each row were harvested individually and used to score the traits of spikelets per panicle, number of primary branches, and number of secondary branches.

Vector construction and genetic transformation

To generate the overexpression vector, coding sequences of OsMFT1 were isolated from ZH11 leaf cDNA and cloned into T-vector (Promega), OsMFT1, OsMFT2, OsMFT3, and OsMFT4, were from previous studies (Xue et al., 2008; Weng et al., 2014); the ghd7 mutant had an SNP mutation resulting in a premature stop codon in the ZH11 background. For measurement of the agronomic traits, rice plants grown at Wuhan were under natural LD conditions, whereas plants grown at Hainan were under SD conditions. Germinated seeds were sown in the seed beds and 1-month-old seedlings were transplanted to the fields with 10 plants in a row. The heading date was the day when the first panicle of the plant emerged. Plants in the middle of each row were harvested individually and used to score the traits of spikelets per panicle, number of primary branches, and number of secondary branches.

RNA extraction and qRT-PCR analysis

Samples of leaves, young panicles, and other tissues were frozen in liquid nitrogen immediately after being collected from the plants. Total RNA was extracted using Trizol reagent (TransGen Biotech, Beijing). Then 3 μg of total RNA was digested by DNase I and reverse transcribed by Superscript II reverse transcriptase (Invitrogen, USA) to obtain the first-strand cDNA according to the manufacturer’s protocol. Real-time PCR was performed in a 96-well plate in an ABI Prism 7500 real-time PCR system (Applied Biosystems, USA) using SYBR Premix ExTaq reagent (TaKaRa, Dalian). The relative expression levels were calculated according to the method proposed previously (Livak and Schmittgen, 2001), with the rice ubiquitin gene serving as an internal control. Primers used for real-time PCR are listed in Supplementary Table S1 at JXB online.

Subcellular localization

To confirm the subcellular localization of OsMFT1, the coding sequence of OsMFT1 was amplified and inserted into the pM999 vector driven by the CaMV 35S promoter. The fusion construct 35S::OsMFT1::YFP was co-transformed into rice protoplasts with 35S::GHD7::CFP, which was used as a nuclear marker. The construct 35S::YFP was used as a control. Rice protoplasts transformation was conducted as previously described (Xie and Yang, 2013). After transformation into rice protoplasts and incubation in the dark for 12–16 h, the fluorescence was observed by confocal microscopy (Leica Microsystems).

Yeast one-hybrid assay

AD-OsLFL1 was constructed by inserting the coding sequence of OsLFL1 into the vector pB42AD (Clontech, USA). The promoter fragments of OsMFT1 were cloned into the vector pLacZ2μ to construct pro::LacZ. The yeast one-hybrid assay was performed as previously described (Tang et al., 2012). Briefly, the AD-OsLFL1 and pro::LacZ were co-transformed into yeast strain EGY48 and spread on the selective medium SD/–Trp/–Ura (Clontech). The grown transformants were transferred to SD/–Trp/–Ura medium containing raffinose, galactose, and X-gal (Sigma-Aldrich, USA) for developing the blue color.

Electrophoretic mobility shift assay

To get the OsLFL1 protein, the coding sequence of OsLFL1 was amplified and cloned into the expression vector pSPUTK (Smaczniak et al., 2012). Proteins were synthesized using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega). The oligonucleotides were synthesized and labeled with 5’-biotin by the Shanghai Sangon Company. Double-stranded oligonucleotides were generated by mixing equal amounts of the complementary single-stranded oligonucleotides and heating for 2 min at 95 ºC, then cooling down to 25 ºC. Biotin-labeled probes were incubated with the OsLFL1 protein in the binding buffer [10 mM Tris (pH 7.5), 50 mM KCl, 1 mM EDTA, 5 mM MgCl2, 1 mM DTT, 50 ng μl−1 Poly (dI–dC), 2.5% glycerol and 0.05% NP-40] for 20 min at room temperature. For the competition reaction, 10-, 20-, 50-, and 100-fold non-labeled probes were mixed with the labeled probes. The reaction mixture was loaded onto a 6% native polyacrylamide gel and run at 4 ºC. The DNA shift was detected by developing the biotin signal using the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions.

Dual luciferase transentional activity assay in rice protoplasts

To test the transcriptional activity of the OsLFL1 protein, the coding sequence of OsLFL1 was fused in-frame with the GAL4 DNA-binding domain GAL4BD as the effector vector, with CaMV35S–Gα4–LUC as the reporter. To test the transcriptional activation activity of OsLFL1 on OsMFT1, the coding sequence of OsLFL1 was driven by CaMV35S as an effector with the luciferase driven by the promoter of OsMFT1 as a reporter. The effectors and corresponding reporters were co-transformed into rice protoplasts with the internal control vector CaMV35S–LUC as previously described (Xie and Yang, 2013). The Dual-Luciferase Reporter Assay System (Promega) was used to measure the luciferase activity. Briefly, the rice protoplasts were lysed with Passive Lysis buffer after incubation overnight. The supernatant of the lystate was incubated with luciferase assay substrate and the firefly luciferase (fLUC) activity was measured with the TECAN Infinite M200 System. After the measurement of fLUC, Stop & Glo substrate buffer was added to the reaction and then the Renilla luciferase (rLUC) activity was measured. Three independent transformations for each combination were performed, and the relative luciferase activity was calculated by the ratio fLUC/rLUC.

RNA in situ hybridization

The probes for hybridization were amplified from the OsMFT1 coding sequence using specific primers and inserted into the pGEM-T vector.

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(Promega) for RNA transcription in vitro. The respective sense and anti-sense probes were produced using SP6 and/or T7 transcriptase labelled with the Digoxigenin RNA labelling kit (Roche). Young panicle tissues were collected and fixed in FAA solution (50% ethanol, 5% acetic acid and 3.7% formaldehyde) at 4 °C overnight. RNA in situ hybridization and immunological detection were performed as previously described (Zhao et al., 2009).

Seed germination test

ZH11 and the transgenic homozygous OX-OsMFT1 lines were grown during normal growing seasons in Wuhan. We marked the panicles when they appeared from the leaf sheath, then harvested the panicles 40 d after their heading. After being dried under sunlight for 3 d, grains were threshed and used for a germination test. Fully filled grains were spread on plates with wet filter paper and immediately moved into an incubator in the dark at 28 °C. Each plate was filled with 50 seeds, and three plates were used for each genotype. Germination was defined as the emergence of the radical, and the number of germinated seeds was counted every half-day after imbibition.

Results

OsMFT1 overexpression and knockout plants showed altered heading date and panicle architecture in rice

To identify the function of OsMFT1, we overexpressed OsMFT1 using the CaMV35S promoter in a japonica variety Zhonghua 11 (ZH11) and obtained 50 T0 transgenic plants, of which 18 were positive (see Supplementary Fig. S1). The positive plants showed delayed heading date and increased spikelet number per panicle (Fig. 1C, D). Overexpression lines had greatly increased number of branches, especially secondary branches (Fig. 1E), resulting in dense panicles. Three lines of OsMFT1 knockout mutants each having a 1 bp deletion, and 1 bp and 2 bp insertion in the first exon were generated using a CRISPR–Cas9 strategy (Supplementary Fig. S2). A slight but significant promotion in heading date and decrease of spikelets per panicle were observed in all OsMFT1 knockout mutants compared with WT (Fig. 1F, I). The number of primary branches and secondary branches was significantly reduced (Fig. 1J). Taken together, OsMFT1 is a suppressor of heading and positive regulator of spikelets per panicle in rice.

Expression characterization of OsMFT1 and subcellular localization

To characterize the spatial–temporal expression pattern of OsMFT1, the RNA transcript level of OsMFT1 was examined in roots, stems, leaves, sheaths, and developing young panicles using quantitative real-time PCR. OsMFT1 was preferably expressed in stem, leaves, sheath, and developing young panicles (Fig. 2A). RNA in situ hybridization revealed that OsMFT1 was slightly expressed in the shoot apical meristem and inflorescence meristem (Fig. 2B, C), and strongly expressed in the primary branch meristem (Fig. 2D), secondary branch meristem (Fig. 2E) and spikelet meristem (Fig. 2F). To determine the subcellular localization of OsMFT1, the full length coding sequence (CDS) of OsMFT1 was fused to the yellow fluorescent protein (YFP) reporter gene driven by the CaMV 35S promoter. Then, the OsMFT1-YFP and GHD7-CFP plasmids were co-transformed into protoplasts. The OsMFT1-YFP fusion protein was luminescent in the nucleus and the YFP fluorescence overlapped with cyan fluorescent protein (CFP) fluorescence, which indicated that OsMFT1 co-localized with GHD7, a nuclear protein (Fig. 2B). Thus, OsMFT1 is a nuclear protein.

The role of OsMFT1 in regulating heading date

OsMFT1 overexpression lines and wild type were used to examine the transcriptional level of key genes involved in the photoperiodic flowering pathway. There were no significant differences in the expression of Ghd7 and Hd1 between OsMFT1 overexpression lines and wild type, while the expression levels of Ehd1, Hd3a, RFT1, and MADS14 were greatly suppressed in overexpression lines (Fig. 3A–G), indicating that OsMFT1 acted upstream of Ehd1. To further elucidate the regulatory relationship between OsMFT1 and these genes, the RNA expression level of OsMFT1 in an Ehd1 overexpression line (OX–Ehd1), a Ghd7 overexpression line (OX–Ghd7), a Ghd7 mutant line (gld7), and a pair of Ghd7 near isogenic lines was examined. Compared with the wild type ZH11, the expression level of OsMFT1 did not vary in the OX–Ehd1 line, indicating that Ehd1 did not regulate OsMFT1 in turn (Fig. 3G). The expression level of OsMFT1 was approximately 4-fold more than that in the OX–Ghd7 line and was reduced by 3-fold in the Ghd7 mutant compared with wild type (Fig. 3G). Similarly, the expression level of OsMFT1 in NIL (mh7) was 3-fold of that in NIL (zs7), which has completely lost Ghd7 (Fig. 3H). In general, OsMFT1 expression is regulated by Ghd7 but not by Ehd1 and, in contrast, OsMFT1 suppressed Ehd1 expression. The expression of other flowering genes was also examined in OsMFT1 overexpression lines and wild type, but no significant differences were detected (see Supplementary Fig. S3). Thus, it is suggested that OsMFT1 acts downstream of Ghd7 and upstream of Ehd1 in the photoperiodic flowering pathway.

OsMFT1 overexpression rescued the phenotype of Ami-Ghd7

To understand the effect of OsMFT1 on Ghd7-mediated flowering and panicle architecture, a hybrid F1 was generated by crossing OX–OsMFT1 with Ami-Ghd7 (Ghd7 artificial microRNA), in which Ghd7 expression was largely suppressed. Four genotypes showing higher OsMFT1 and lower Ghd7 (Ami-Ghd7/OX–OsMFT1), higher OsMFT1 but normal Ghd7 (OX–OsMFT1), lower Ghd7 but normal OsMFT1 (Ami-Ghd7) and wild type were identified from an F2 population (see Supplementary Fig. S4). The heading date and spikelets per panicle of four genotypes in long-day conditions are displayed in Fig. 4. Compared with the wild type plants, Ami–Ghd7 showed significantly advanced heading date and smaller panicle size while the OsMFT1 overexpression line
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OsLFL1 activated OsMFT1 expression by directly binding to its promoter

In Arabidopsis, ABI3 directly binds to the RY motif in the promoter of AtMFT (Park et al., 2011; Mao and Sun, 2015). Among the ABI3 homologs reported in rice, OsLFL1 is a flowering repressor (Peng et al., 2008; Romanel et al., 2009). Thus, OsLFL1 is proposed to probably bind to OsMFT1 promoter directly. Then, the 1.6 kb sequence of the OsMFT1 promoter and 5′-UTR region was divided into four fragments and used to perform a yeast one-hybrid assay with OsLFL1 protein. A binding activity of OsLFL1 protein to the fourth fragment (pro4) closest to ATG was identified (Fig. 5A, B). cis-Element analysis identified an RY motif (CATGCATG) 221 bp upstream of the translation start site ATG in the OsMFT1 promoter (Fig. 5A). An electrophoretic mobility shift assay (EMSA) showed that OsLFL1 protein directly bound to the 50-bp fragment containing the RY motif in vitro (Fig. 5C).

To verify how OsLFL1 regulates OsMFT1, a dual-luciferase transient assay was performed in rice protoplasts to examine the transcriptional activity of OsLFL1. As shown in Fig. 5D, E, with the firefly luciferase driven by five copies of the yeast GAL4 binding domain (GAL4BD) as a reporter, relative luciferase activity of OsLFL1 fused with GAL4BD as an effector was much higher than GAL4BD itself as an effector, indicating that OsLFL1 had significant transcriptional activation activity; with the firefly luciferase driven by the OsMFT1 promoter as an effector, relative luciferase activity of OsLFL1 as an effector was 2-fold of empty ‘none’ as an effector indicating that OsLFL1 had activation activity on the OsMFT1 promoter. Taken together, OsLFL1 activates OsMFT1 expression by directly binding to its RY motif in the promoter.

Overexpression of OsMFT1 suppressed the expression of spikelet meristem identity genes

To identify downstream genes of OsMFT1, the 0.5–1 mm young panicles from OX-OsMFT1 and wild type plants were used for transcriptome sequencing to find differentially expressed genes. Compared with wild type, 84 genes were up-regulated and 86 genes were down-regulated in the OX-OsMFT1 young panicles (see Supplementary Table S2). Three genes regulating spikelet meristem differentiation were down-regulated, including MADS1, MADS5, and FZP. FZP is a spikelet meristem identity gene that determines the transition from panicle branching to spikelet formation. OsMADS1 and OsMADS5, together with OsMADS7, OsMADS8, and OsMADS34 are five SEPALLATA-like genes that are classified as class E genes for floral determinacy (Cui et al., 2010). A qRT-PCR assay showed that these genes were indeed down-regulated in OX-OsMFT1 (Fig. 6A). Further RNA in situ hybridization showed that the significantly weaker expression of FZP, OsMADS1, and OsMADS8 was detected in the secondary branch meristem of OX-OsMFT1 compared with wild type (Fig. 6B–D).
Discussion

Conserved functions in regulating flowering and panicle architecture between MFT-like and TFL1-like families in rice

Most of the PEBP family genes are functionally conserved among higher plants. Previous studies indicated that FT-like genes generally induce flowering both in monocots and dicots, while TFL1-like genes delay flowering and regulate the inflorescence architecture, including TFL1 in Arabidopsis, RCNs in rice, and ZCNs in maize (Alvarez et al., 1992; Nakagawa et al., 2002; Zhang et al., 2005; Danilevskaya et al., 2010; Wickland and Hanzawa, 2015). As the evolutionary ancestor of FT and TFL1, MFT-like genes are generally related to seed germination (Xi et al., 2010; Nakamura et al., 2011; Li et al., 2014; Hu et al., 2016). As expected, OsMFT1 also regulates seed germination, which will be further studied in future (see Supplementary Fig. S6). OsMFT1 overexpression significantly delays flowering and largely increases branching, and the OsMFT1 knockout mutant promotes flowering and reduces branching. The performance of OsMFT1 overexpression plants is very similar to rice TFL1-like gene (RCN1, RCN2, and RCN3) overexpression plants (Nakagawa et al., 2002; Zhang et al., 2005). In addition, knocking down four TFL1-like genes in rice resulted in small panicles with reduced branches, similar to the OsMFT1 knockout plants (Liu et al., 2013). We examined the expression of rice TFL1-like genes (RCN1, RCN2, and RCN3) in the leaves and panicles of OsMFT1 overexpression lines and wild
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type, and no big differences were detected (Supplementary Fig. S7). Since OsMFT1 and rice TFL1-like genes have similar functions in regulating flowering and inflorescence architecture, functional redundancy may exist between them.

FT and TFL1 have only 39 non-conservative amino acid substitutions but have distinct functions due to a potential ligand binding residue and a divergent external loop (Hanzawa et al., 2005; Ahn et al., 2006). Lateral studies revealed that the mutation of at least four residues converts FT into a complete TFL1 mimic by affecting the protein surface charge through testing the effects of numerous mutations of FT in vivo (Ho and Weigel, 2014). We compared the crucial amino acid residues of FT-like and TFL1-like protein in both Arabidopsis and rice (see Supplementary Fig. S8); furthermore, we analyzed the potential function of OsMFT1 according to the reported mutations in FT and their corresponding phenotypes (Ho and Weigel, 2014). The results indicate that the key amino acid residues of FT-like and TFL1-like proteins are conserved between Arabidopsis and rice, and the methionine (M) at position 109, lysine (K) at 128 and alanine (A) at 138 most likely contribute to the conferred TFL1 activity of OsMFT1 (Supplementary Table S3), which could explain the functional similarity of OsMFT1 and TFL1-like genes in rice.

In Arabidopsis, MFT mainly functions in regulating seed germination instead of flowering (Yoo et al., 2004; Xi et al., 2010). In this study, OsMFT1 had effects on both seed germination and flowering (Fig. 1; Supplementary Fig. S6). It is noted that the upstream regulator Ghd7 and downstream gene Ehd1 of OsMFT1 are specific genes in rice that have no homologs identified in Arabidopsis (Doi et al., 2004; Xue et al., 2008). This is probably why MFT1 has no regulation in flowering in Arabidopsis.

Activation of OsMFT1 by both OsLFL1 and Ghd7

A previous study has proposed that Ghd7 positively regulates OsMFT1 in flowering through an eQTL-guided
function-related co-expression analysis (Wang et al., 2014). Here, we have further demonstrated that Ghd7 regulates OsMFT1 expression transcriptionally and OsMFT1 plays a role in regulating flowering time and spikelets per panicle downstream of Ghd7 using double mutants. Ghd7 is a central regulator that has many downstream targets to regulate multiple traits (Weng et al., 2014), and OsMFT1 is only one of the downstream targets of Ghd7. That is probably why Ami-Ghd7/OX-MFT1 did not fully show the performance of OX-OsMFT1 and exhibited an intermediate phenotype. Both OsLFL1 and GHD7 are transcriptional factors activating OsMFT1 expression at transcriptional level. OsLFL1 directly binds to the promoter of OsMFT1 (Fig. 5), but yeast one-hybrid assay demonstrated that GHD7 didn’t bind to the OsMFT1 promoter (see Supplementary Fig. S9), indicating that GHD7 probably regulates OsMFT1 expression indirectly. Recent studies have revealed that an RY motif in FLC and EU1 could recruit a complex for gene repression (Yuan et al., 2016; Xie et al., 2018). It is possible that an RY motif in OsMFT1 recruits a protein complex consisting of OsLFL1 and GHD7 for gene activation, or one of the Ghd7 downstream targets also directly binds to the promoter of OsMFT1.

**Prolonged branch meristem differentiation caused dense panicles in OsMFT1 overexpression plants**

Rice panicle is derived from the inflorescence meristem, and the inflorescence meristem develops branch meristems. The differentiation of spikelet meristems indicates that the branch meristem stops producing branch primordia and terminates to be a spikelet. Here, we found that overexpressing OsMFT1 significantly represses FZP and SEPALLATA-like genes expression. Repression or absence of FZP causes the branch meristem to produce more lateral branch primordia instead of spikelet primordia and overexpression of FZP accelerates the spikelet formation, which results in fewer branches (Komatsu et al., 2003; Bai et al., 2016). The latest reports on FZP have revealed that transcriptional silencer-mediated repression of FZP expression increases spikelet number per panicle (Bai et al., 2017). Class E genes are required for floral determinacy, including the five SEPALLATA-like genes OsMADS1, OsMADS5, OsMADS7, OsMADS8, and OsMADS34. Ectopic expression of OsMADS1 was reported to cause reduced branches and small panicles (Wang et al., 2017). Thus the delay of accumulation of RNA expression of FZP and SEPALLATA-like genes indicates the delay of acquiring spikelet meristem identity. Hence, we proposed OX-OsMFT1 has prolonged branch meristem differentiation, which leads to increased branches and spikelets. Nevertheless, the mechanism of OsMFT1 regulation of FZP and SEPALLATA-like genes is still unknown.

Based on these results, we propose a working model of OsMFT1 (Fig. 7). OsLFL1 protein directly binds to the OsMFT1 promoter and activates OsMFT1 expression. OsMFT1 negatively regulates flowering downstream of Ghd7 and upstream of Ehd1. Meanwhile, OsMFT1 represses the
**Fig. 5.** In vivo and in vitro assay of OsLFL1 binding to the promoter of OsMFT1. (A) The promoter of OsMFT1 was divided into four fragments (pro1–4), and the RY motif was contained in pro4. (B) OsLFL1 bound to pro4 in yeast cells through a yeast one-hybrid assay on selective medium (SD/−Trp−Ura) containing X-gal for developing the blue color. (C) EMSA assay using the OsLFL1 protein and 50-bp OsMFT1 promoter containing RY motif as a probe labeled with 5′-biotin. The 10-, 20-, 50- and 100-fold non-labeled probes were used for competition. (D, E) OsLFL1 activates the expression of OsMFT1 by dual luciferase transient assay in rice protoplasts. Error bars indicate SD based on three biological replicates, **P<0.01, t-test. (This figure is available in color at JXB online.)

**Fig. 6.** Differential expression of rice SEP-like genes and FZP between OsMFT1 overexpression lines and wild type. (A) RNA expression comparison of FZP, OsMADS1, OsMADS5, OsMADS7, OsMADS8, and OsMADS34 between OX-OsMFT1 and wild type using quantitative real time PCR. Error bars indicate SD based on three biological replicates. (B–D) RNA in situ hybridization of FZP (B), OsMADS1 (C), and OsMADS8 (D) in young panicles of wild type (left) and OsMFT1 overexpression lines (right). Scale bars, 100 µm. Red arrowheads show secondary branch meristems. (This figure is available in color at JXB online.)
expression of genes for spikelet meristem identity and prolongs branch differentiation, which results in more panicle branches. Hence, OsMFT1 is a suppressor of heading and a positive regulator of panicle architecture in rice.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. RNA expression level of partial T0 individuals of OX-OsMFT1.

Fig. S2. The mutation positions and mutation types of three OsMFT1 CRISPR lines.

Fig. S3. RNA expression level comparison of some rice flowering genes.

Fig. S4. Expression of Ghd7 and OsMFT1 in four genotypes from an F2 population.

Fig. S5. Comparison of heading date and panicle architecture among four genotypes.

Fig. S6. Comparison of germination speed between ZH11 and OX-OsMFT1 lines.

Fig. S7. RNA expression level comparison of rice TFL1-like genes between ZH11 and OX-OsMFT1 lines.

Fig. S8. Amino acid alignment of FT-like and TFL1-like protein segments in Arabidopsis and rice.

Fig. S9. Yeast one-hybrid assay of GHD7 and OsMFT1 promoter.

Table S1. Primers used in this study.

Table S2. Differentially expressed genes between ZH11 and OX-OsMFT1 young panicles.

Table S3. Comparison of six important amino acid residues between AtFT, AtTFL1, and OsMFT1.

Acknowledgements

We thank the farm technician Mr. J. B. Wang for his excellent work in the field. This work was funded by the National Natural Science Foundation of China (31571751), National Special Program for Research of Transgenic Plant of China (2011ZX08009-001-002) and National Key Research and Development Program of China (2016YFD0100301).

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