

# Overexpression of *RICE FLOWERING LOCUS T 1 (RFT1)* Induces Extremely Early Flowering in Rice

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RICE FLOWERING LOCUS T 1 (RFT1) is a major florigen that functions to induce reproductive development in the shoot apical meristem (SAM). To further our study of RFT1, we overexpressed the gene and examined the expression patterns of major regulatory genes during floral transition and inflorescence development. Overexpression induced extremely early flowering in the transgenics, and a majority of those calli directly formed spikelets with a few spikelets, thus bypassing normal vegetative development. FRUITFULL (FUL)-clade genes *OsMADS14*, *OsMADS15*, and *OsMADS18* were highly induced in the RFT1-expressing meristems. *OsMADS34* was also induced in the meristems. This indicated that RFT1 promotes the expression of major regulatory genes that are important for inflorescence development. RFT1 overexpression also induced SEPALLATA (SEP)-clade genes *OsMADS1*, *OsMADS5*, and *OsMADS7* in the greening calli before floral transition occurred. This suggested their possible roles at the early reproductive stages. We found it interesting that expression of *OsFD1* as well as *OsFD2* and *OsFD3* was strongly increased in the RFT1-expressing calli and spikelets. At a low frequency, those calli produced plants with a few leaves that generated a panicle with a small number of spikelets. In the transgenic leaves, the FUL-clade genes and *OsMADS34* were induced, but SEP-clade gene expression was not increased. This indicated that *OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS34* act immediately downstream of RFT1.

**Keywords:** florigen, flowering, FRUITFULL, OsFD1, RFT1, SEPALLATA

## INTRODUCTION

Flowering plants undergo a transition between the vegetative and reproductive stage. In rice (*Oryza sativa*), once flowering is triggered, the shoot apical meristem (SAM) is converted into the rachis meristem (RM), which produces the first bract primordium at the opposite side of the flag leaf. This RM generates the primary branch meristem (PBM) in the axils of those newly developed bracts. During primary branch elongation, the PBM produces a secondary branch meristem (SBM). Both PBM and SBM are eventually converted to a terminal spikelet meristem, which then forms a single floral meristem (FM) that then successively produces floral organs (Itoh et al., 2005).

Reproductive development is triggered by the accumulation of florigens in the leaf phloem (Komiya et al., 2008; Tamaki et al., 2007). In rice, *Heading date 3a (Hd3a)* and *RICE FLOWERING LOCUS T1 (RFT1)* are florigen genes that dominate under short-day and long-day conditions, respectively (Komiya et al., 2008; Tamaki et al., 2015). They are expressed preferentially in phloem cells when flowering signal is induced developmentally or environmentally (Cho et al., 2017). The 200-bp region between -245 bp and -45 bp from the transcription initiation site of *Hd3a* is responsible for this phloem-specific expression (Pasriga et al., 2018).

Expression of the rice florigen genes is promoted by a type-B responsive regulatory element Ehd1 after the formation of a homodimer (Cho et al., 2016). Homodimerization of the protein is inhibited by cytokinin-inducible OsRR1.

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Transcription of *Ehd1* is suppressed by several upstream regulatory elements, such as *Ghd7*, *Hd1*, *COL4*, *AP2*, and *OsLFL1*, but is induced by positive elements, including *OsID1*, *Ehd4*, *OsMADS51*, and *Hd1* (Cho et al., 2017; Lee and An, 2015; Tsuji et al., 2011).

*Hd3a* and *RFT1* proteins are transferred to the SAM, where they activate downstream target genes to initiate reproductive processes. *Hd3a* protein interacts with 14-3-3 protein of the *Gf14* family, which mediates the interaction between *Hd3a* and *OsFD1*, thus forming a ternary complex that is targeted to the nucleus (Taoka et al., 2011; Tsuji et al., 2013). This 'florigen activation complex (FAC)' stimulates expression of *OsMADS14*, *OsMADS15*, and *OsMADS18*, all *FRUITFULL (FUL)*-clade MADS box genes, as well as expression of *OsMADS34*, a *SEPALLATA (SEP)*-clade gene (Kobayashi et al., 2012; Taoka et al., 2011). The complex binds with the *OsMADS15* promoter to induce expression of the target gene (Tsuji et al., 2011). Expression of *OsMADS14* and *OsMADS15* is decreased in the SAM of *Hd3a* RNAi or *RFT1* RNAi plants, which indicates that they are downstream genes of the florigens (Komiya et al., 2008, 2009; Tsuji et al., 2011).

Florigens also function outside of the SAM. For example, *Hd3a* protein is accumulated in axillary meristems and forms an FAC to promote branching (Tsuji et al., 2015). This florigen induces outgrowth by lateral buds through a mechanism independent of strigolactone. Both *Hd3a* and *RFT1* also form transcriptional activation or repression complexes in rice leaves, where the proteins interact with *Gf14c* and *OsFD1* (Brambilla et al., 2017). Transient induction of *Hd3a* and *RFT1* expression induces transcript levels of *OsMADS14* and *OsMADS15* within 16 h (Brambilla et al., 2017). In addition, overexpression of *OsFD1* increases transcript levels of *OsMADS14* and *OsMADS15* as well as those of *Ehd1*, *Hd3a*, and *RFT1* in the leaves (Brambilla et al., 2017).

Suppression of all three *FUL*-clade genes -- *OsMADS14*, *OsMADS15*, and *OsMADS18* -- slightly delays the reproductive transition. However, further depletion of *OsMADS34* from triple knockdown plants significantly delays that transition, which means that all four of those genes coordinately act in the SAM to induce reproductive development (Kobayashi et al., 2012). *OsMADS34* is induced in the SAM upon transition to reproductive development and also while glumes are initiated during spikelet development (Kobayashi et al., 2012). This observation is consistent with genetic information that mutations in the gene causes reduced primary branches and altered glume morphology (Gao et al., 2010; Kobayashi et al., 2012). *OsMADS34* interacts with *OsMADS14* and *OsMADS15*, which suggests a possibility that they form a ternary complex to induce downstream genes (Kobayashi et al., 2012). *OsMADS34* also functions together with *OsMADS1* and *OsMADS5* in determining spikelet identity (Wu et al., 2018).

Mis-expression of *Hd3a* markedly accelerates the reproductive transition and produces terminal tissues at the tips of transgenic rice plants (Izawa et al., 2002; Kojima et al., 2002). The terminal tissues often contain a floret containing several stamen-like and a pistil-like organ. In severe cases, spikelets develop directly from calli (Kobayashi et al., 2012).

When *Hd3a* is expressed in *osmasd34* mutant plants, the transgenic plants produced elongated stems, which implies a transition to reproductive development (Kobayashi et al., 2012). However, they do not develop inflorescence meristems and, instead, repeatedly generate vegetative shoots that result in a bushy appearance. This suggests that *OsMADS34* is needed for initiation of inflorescence development.

In this study we observed that overexpression of *RFT1* resulted in the direct formation of spikelets from most of the transgenic calli. Transcript analyses at different developmental stages indicated that expression of *OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS34* in those calli was strongly induced during the reproductive phase. In addition, *OsMADS1*, *OsMADS5*, and *OsMADS7* were transiently induced in greening calli before that phase.

## MATERIALS AND METHODS

### Plant growth and sampling

Rice plants (var. japonica; 'Nipponbare') were grown in a paddy field at Yongin, Korea. Shoot apices containing vegetative SAMs were sampled at two stages: V1, approximately 70 days after sowing (DAS); and V2, at 73~76 DAS. The transition stage V/R occurred at approximately 79 DAS. Shoot apical regions containing reproductive SAMs were isolated at four stages, as described by Tamaki et al. (2015). The R1 stage was at approximately 82 DAS, when the first bract was formed; R2, at approximately 85 DAS, when PBM development was initiated; R3, at approximately 88 DAS, when PBMs were elongating; and R4, at approximately 91 DAS, when SBM development was initiated. We also sampled inflorescences at three stages, as described by Itoh et al. (2005). Stage In6 indicated the time at which the inflorescences were 1.5 mm long while spikelet development was beginning; In7, inflorescences approximately 20 mm long, and floral organs starting to form; and In8, panicles approximately 200 mm long and reproductive organs now mature.

### Vector construction and rice transformation

For generation of the *RFT1*-overexpression construct, the 537-bp full length cDNA was amplified by PCR using mRNA prepared from japonica rice ('Dongjin') and a pair of primers (CGCAAGCTTATGGCCGGCAGCGGCAGGGA and GCACTA G TCTAGGGGTAGACCCTCTGC, where underlined sequences are *HindIII* and *SpeI* enzyme sites, respectively). The PCR fragment was cloned into the pGA3426 binary vector under the control of the maize *ubiquitin 1* promoter using the restriction enzyme sites (Kim et al., 2009). After checking its quality by DNA-sequencing, we transformed the construct into *Agrobacterium tumefaciens* LBA4404. Transgenic rice plants were generated by the stable transformation method, as previously reported (An et al., 1988). The putative transgenics were confirmed via selection on hygromycin and transferred to shoot induction media containing 40 mg L<sup>-1</sup> hygromycin, 2.5 mg L<sup>-1</sup> kinetin, and 0.1 mg L<sup>-1</sup> NAA.

### RNA isolation and quantitative RT-PCR analyses

Total RNA was isolated from the samples of various tissue

types using RNAiso Plus (TaKaRa, Japan; <http://www.takarabio.com>), and was quantified by a Nano Nanodrop ND-2000 Spectrophotometer (Thermo Scientific, USA, <http://www.nanodrop.com>), as described previously (Cho et al., 2018). The cDNA was prepared with 10 ng of the oligo (dT)<sub>18</sub> primer, 2.5 mM deoxy ribonucleotide triphosphates, and Moloney murine leukemia virus reverse transcriptase. Quantitative real-time RT-PCR (qRT-PCR) was performed with the synthesized cDNAs as templates and the gene-specific primers (Supplementary Table S1), using SYBR Premix Ex Taq™ II (TaKaRa) and the Rotor-Gene 6000 instrument system (Corbett Research, Sydney, Australia; <http://www.corbettlifescience.com>). Rice *ubiquitin 1 (Ubi1)* served as an internal control, and at least three biological replicates were analyzed. We used the data only when the melting curve showed a single sharp peak.

### Histochemical analysis

Samples of various tissues were fixed in formalin-acetic acid-alcohol (FAA) solution after vacuum-infiltration, as described previously (Yoon et al., 2014; 2017). After incubation overnight at 4°C, the samples were dehydrated through an ethanol series (50, 70, 90, and 100%). They were treated with a tert-butyl alcohol series and paraffin was infiltrated. The fixed tissues were sectioned to 10-µm thickness with a microtome (model 2165; Leica Microsystems, <http://www.leica-microsystems.com/>). After attachment to coated slides, the samples were rehydrated with 100% Histo choice for clearing, using an ethanol series (100, 70, 50, and 30%) and distilled water. They were then stained with toluidine and observed under a BX61 microscope (Olympus, <http://www.olympus-global.com/en/>).

### Statistical analyses

The P values were generated by ANOVA with the Tukey HSD test using the test groups R program (Cohen and Cohen, 2008).

## RESULTS

### Overexpression of *RFT1* induces extremely early flowering

Overexpression of *Hd3a* prompts extremely early flowering (Izawa et al., 2002; Kobayashi et al., 2012; Kojima et al., 2002). However, the roles of *RFT1* when over-expressed have not been as thoroughly investigated. To expand on our knowledge about the functions of that gene, we generated transgenic calli expressing full-length *RFT1* cDNA under the control of the maize *ubiquitin 1* promoter. Among the 30 independently transformed calli, three regenerated leaves and plantlets (Figs. 1A-1C), but did not proceed to mature vegetative stages. Instead, they produced small panicles with only a few spikelets (Figs. 1D-1F). Results from qRT-PCR analyses showed that *RFT1* was expressed in all three transgenic plants, indicating that the early flowering phenotypes were due to its overexpression (Fig. 1G).

We assayed leaf samples to study regulatory genes that are affected by overexpression of *RFT1*. Two FUL-clade MADS box genes, *OsMADS14* and *OsMADS15*, were examined

because they are considered downstream genes of *RFT1* in the SAM (Komiya et al., 2009). Our data indicated that expression levels of both MADS box genes were significantly higher in the transgenic leaves when compared with transcript levels detected in the leaves of control plants that had been transformed with the empty vector (Figs. 1H and 1I). This observation supported our proposal that they are immediate target genes of *RFT1*. Expression of *OsMADS18*, which belongs to the same clade with *OsMADS14* and *OsMADS15*, was also enhanced in the transgenic leaves (Fig. 1J).

We also studied SEP clade genes because some members are associated with the development of inflorescence meristems (Gao et al., 2010; Kobayashi et al., 2012). Transcript levels of *OsMADS34* were much higher in the *RFT1*-expressing leaves than in the control (Fig. 1K). As a key regulatory gene, *OsMADS34* is preferentially expressed in inflorescence meristems and controls the development of inflorescences and spikelets (Kobayashi et al., 2012). In contrast, the transcript level of another SEP clade gene, *OsMADS1*, was decreased in the transgenic leaves (Fig. 1L). Expression levels of the other SEP clade genes -- *OsMADS5*, *OsMADS7*, and *OsMADS8* -- were extremely low in the control leaves, and overexpression of *RFT1* did not significantly alter their expression (Figs. 1M-1O). These findings indicated that *OsMADS34* is the only SEP member that is stimulated in the leaves by *RFT1*. Finally, expression of *OsMADS50*, which is homologous to the *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* in Arabidopsis (Lee et al., 2004), was suppressed in *RFT1*-overexpressing leaves (Fig. 1P).

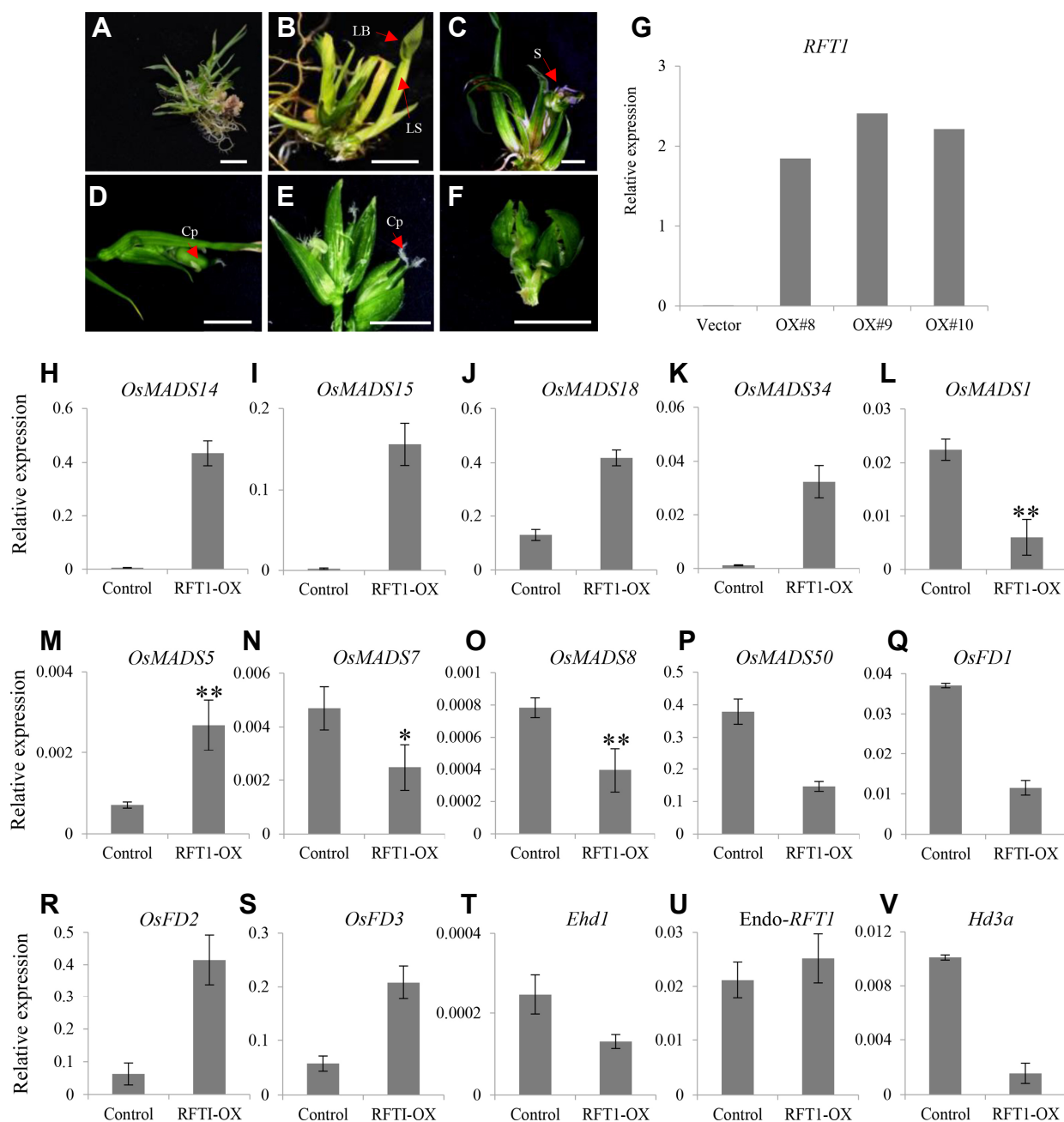
Florigen proteins induce downstream genes together with *OsFD1* (Kobayashi et al., 2012). Analysis of three FD-like genes revealed that expression of *OsFD1* did not differ between *RFT1*-expressing leaves and WT leaves (Fig. 1Q). However, transcript levels of *OsFD2* and *OsFD3* were much higher in those transgenic leaves (Figs. 1R and 1S).

Because transient activation of *Hd3a* and *RFT1* expression in leaves suppresses transcript levels of endogenous *Hd3a*, *RFT1*, and *Ehd1* (Brambilla et al., 2017), we measured their transcript levels to determine whether similar suppression occurred in transgenic leaves that over-express *RFT1*. Our assay results showed that *Ehd1* expression remained at a very low level in those leaves (Fig. 1T). Whereas endogenous *RFT1* expression was also unaltered (Fig. 1U), transcript levels of *Hd3a* were reduced in the *RFT1* leaves (Fig. 1V).

### *RFT1* expression directly induces spikelet development from regenerating calli

All of the remaining 27 calli directly developed into spikelets without plant regeneration (Figs. 2C and 2D). Seven transgenics were selected to analyze expression for the introduced *RFT1*. Here, *RFT1* was expressed at high levels in all of the transformed calli, whereas calli derived from the vector alone as a control did not express the gene (Fig. 2A).

Approximately one month after transfer to shoot induction medium, *RFT1*-overexpressing calli developed leaf-like organs (Fig. 2C) that were significantly different from the leaves developed from WT calli (Fig. 2B). Those leaf-like organs were shorter than normal but the number produced from each callus was higher than that of leaves developing

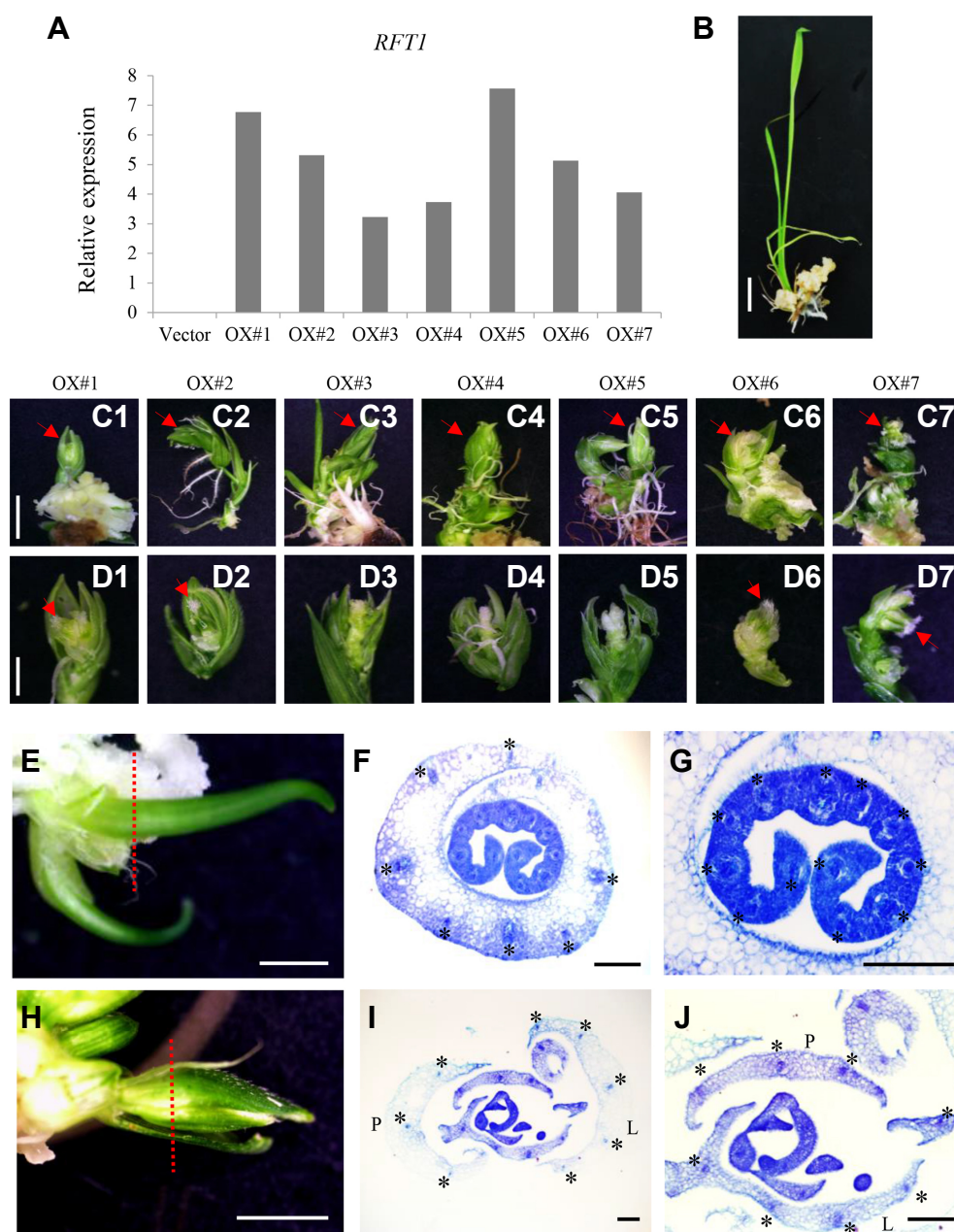


**Fig. 1. Expression analyses of regulatory genes controlling reproductive development in transgenic plants expressing *RFT1*.** (A-C) Three independent plants regenerated from *RFT1*-expressing calli. LB, leaf blades; LS, leaf sheath; S, spikelet. (D-F) Spikelets from three independent plants. (G) Transcript levels of *RFT1* in transgenic plants #8, #9, and #10, compared with control plant transformed with empty vector. Transcript levels of *OsMADS14* (H), *OsMADS15* (I), *OsMADS18* (J), *OsMADS34* (K), *OsMADS1* (L), *OsMADS5* (M), *OsMADS7* (N), *OsMADS8* (O), *OsMADS50* (P), *OsFD1* (Q), *OsFD2* (R), *OsFD3* (S), *Ehd1* (T), endogenous *RFT1* (U), and *Hd3a* (V) in leaves. Expression levels are relative to *OsUbi1*. Error bars indicate standard deviation for 6 biological replicates. Scale bar = 2 mm. \* and \*\* indicate significant difference at  $p < 0.05$  and  $p < 0.01$ , respectively.

from the regenerated WT plants. The leaf-like organs were stacked together to form spikelet-like structures. After rapid generation of multiple leaf-like organs, spikelets appeared at the tops of the shoots (Fig. 2D). Occasionally, multiple car-

pels developed that were often fused to each other (Figs. 2D1, 2D6 and 2D7). Although stamens also formed, they usually numbered fewer than 6 and were morphologically abnormal (Fig. 2D).

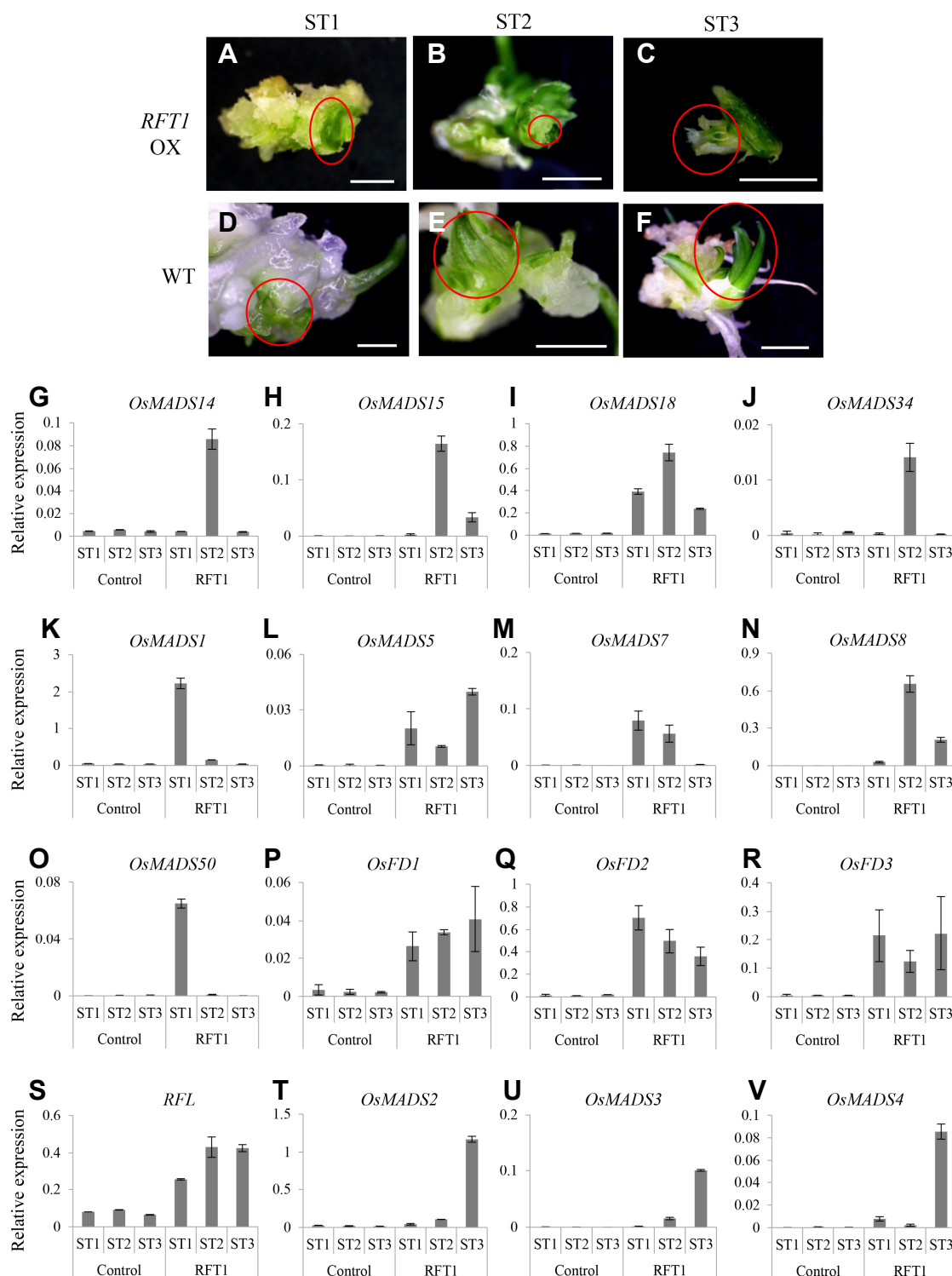




**Fig. 2. Phenotypes of *RFT1*-overexpressing transgenics.** (A) Transcript levels of *RFT1* in 7 independent transgenics (#1 through #7) compared with control plant transformed with empty vector. Expression levels are relative to *OsUbi1*. (B) Regenerating transgenic plant with empty vector. Scale bar = 5 mm. (C) Transgenic plants #1 (C1), #2 (C2), #3 (C3), #4 (C4), #5 (C5), #6 (C6), and #7 (C7). Bars = 5 mm. (D) Close-up of spikelets from #1 (D1), #2 (D2), #3 (D3), #4 (D4), #5 (D5), #6 (D6), and #7 (D7). Bars = 2 mm. (E) Regenerating leaves from control plant transformed with empty vector. Red bar indicates location of cross-cut. (F and G) Cross section of leaves from control plant. Asterisks indicate vascular bundles. (H) Spikelet from *RFT1*-overexpressing transgenic at Stage 3. Red bar indicates location of cross-cut. (I and J) Cross section of spikelet from *RFT1*-overexpressing transgenic. Asterisks indicate vascular bundles. L, lemma; P, palea. Scale bars = 2 mm (E, H) or 100  $\mu$ m (F, G, I, and J).

To investigate the nature of those leaf-like organs, we fixed them in paraffin and observed their cross cut sections under the microscope (Fig. 2H). The samples possessed three or five vascular bundles that resembled palea or lemma, respectively (Figs. 2I and 2J). In contrast, leaves from WT calli

carried more than five vascular bundles, similar to leaves from normal plants (Figs. 2E-2G). This finding indicates that the multiple palea/lemma-like organs arose directly from the *RFT1*-transgenic calli, bypassing the usual vegetative development and branching.

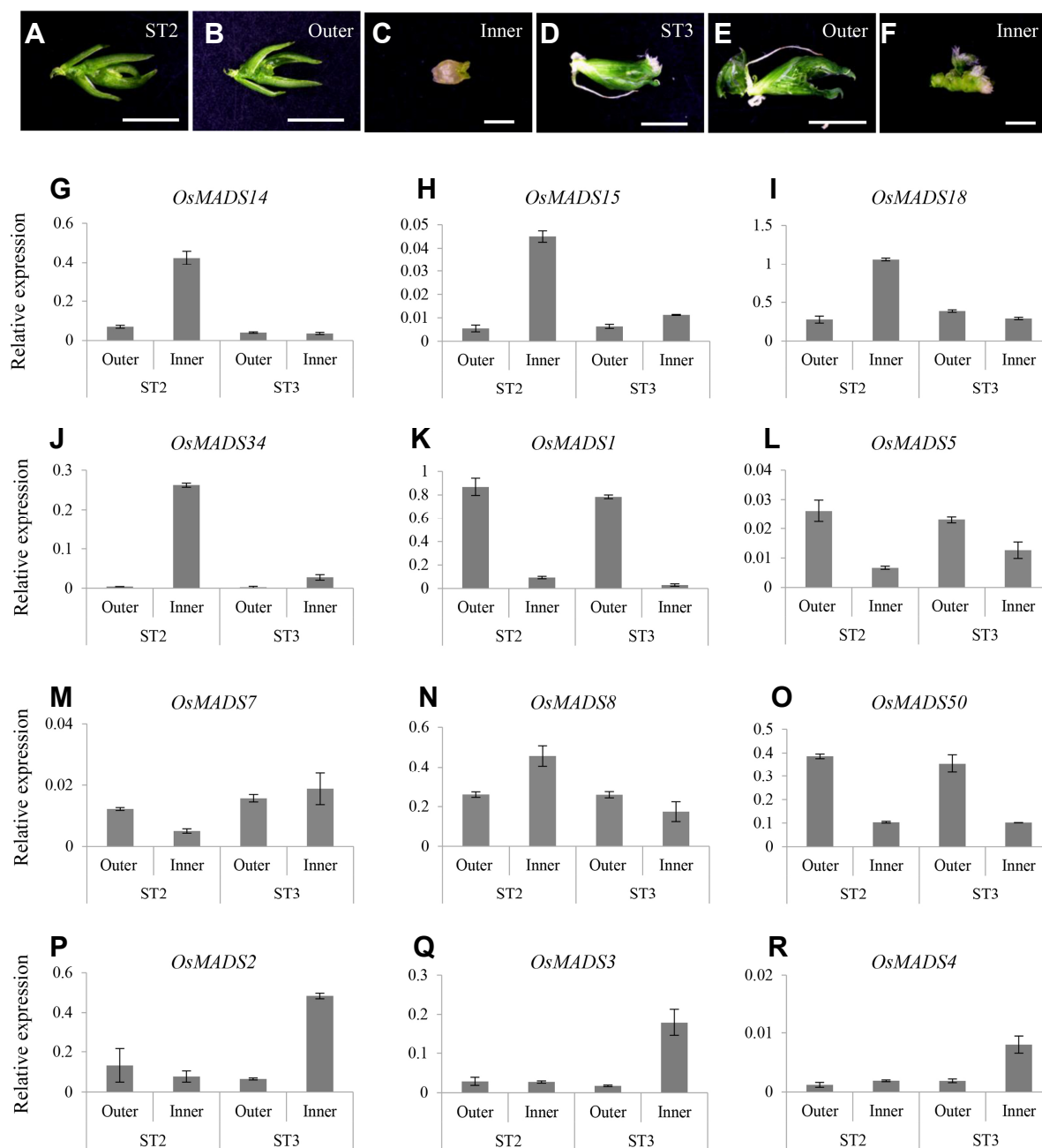


**Fig. 3. Expression analyses of regulatory genes during spikelet development in *RFT1*-overexpressing transgenics compared with WT.** (A) Stage 1. *RFT1*-overexpressing transgenic callus with greening tissues approximately 1 month after shoot induction. (B) Stage 2. Transgenic callus with multiple leaf-like organs, approximately 6 d after Stage 1. (C) Stage 3. Floret inside spikelet from transgenic, approximately 5 d after Stage 2. (D-F) Control plants transformed with empty vector, visualized at stages equivalent to ST1, ST2, and ST3 of *RFT1*-expressing transgenics. Scale bar: 2 mm. Transcript levels of *OsMADS14* (G), *OsMADS15* (H), *OsMADS18* (I), *OsMADS34* (J), *OsMADS1* (K), *OsMADS5* (L), *OsMADS7* (M), *OsMADS8* (N), *OsMADS50* (O), *OsFD1* (P), *OsFD2* (Q), *OsFD3* (R), *RFL* (S), *OsMADS2* (T), *OsMADS3* (U), and *OsMADS4* (V). Expression levels are relative to *OsUbi1*. Error bars indicate standard deviation for 6 biological replicates.

### Expression patterns of regulatory genes during spikelet development from calli

Molecular events during spikelet development from the calli were monitored using RNA extracted from tissues at three different stages: Stage 1, greening undifferentiated tissues at approximately one month after shoot induction (Fig. 3A);

Stage 2, meristem tissues covered by the palea/lemma-like organs at approximately 6 d after Stage 1 (Fig. 3B); and Stage 3, florets with carpels and stamens at approximately 5 d after Stage 2 (Fig. 3C). We collected WT samples at similar stages even though they had differentiated to leaves rather than spikelets (Figs. 3D-3F).



**Fig. 4. Expression analyses of regulatory genes in inner and outer organs of spikelet developed from *RFT1*-overexpressing transgenics.** (A) Spikelet at Stage 2. (B) Outer parts of spikelet at Stage 2. (C) Meristem tissue located inside spikelet at Stage 2. (D) Spikelet at Stage 3. (E) Outer parts of spikelet at Stage 3. Scale bar: 2 mm (A, B, D, E, and F) or 1 mm (C). (F) Meristem tissue located inside spikelet at Stage 3. (G-R) Transcript levels of *OsMADS14* (G), *OsMADS15* (H), *OsMADS18* (I), *OsMADS34* (J), *OsMADS1* (K), *OsMADS5* (L), *OsMADS7* (M), *OsMADS8* (N), *OsMADS50* (O), *OsMADS2* (P), *OsMADS3* (Q), and *OsMADS4* (R) at 2 different stages. Expression levels are relative to *OsUbi1*. Error bars indicate standard deviation for 6 biological replicates.

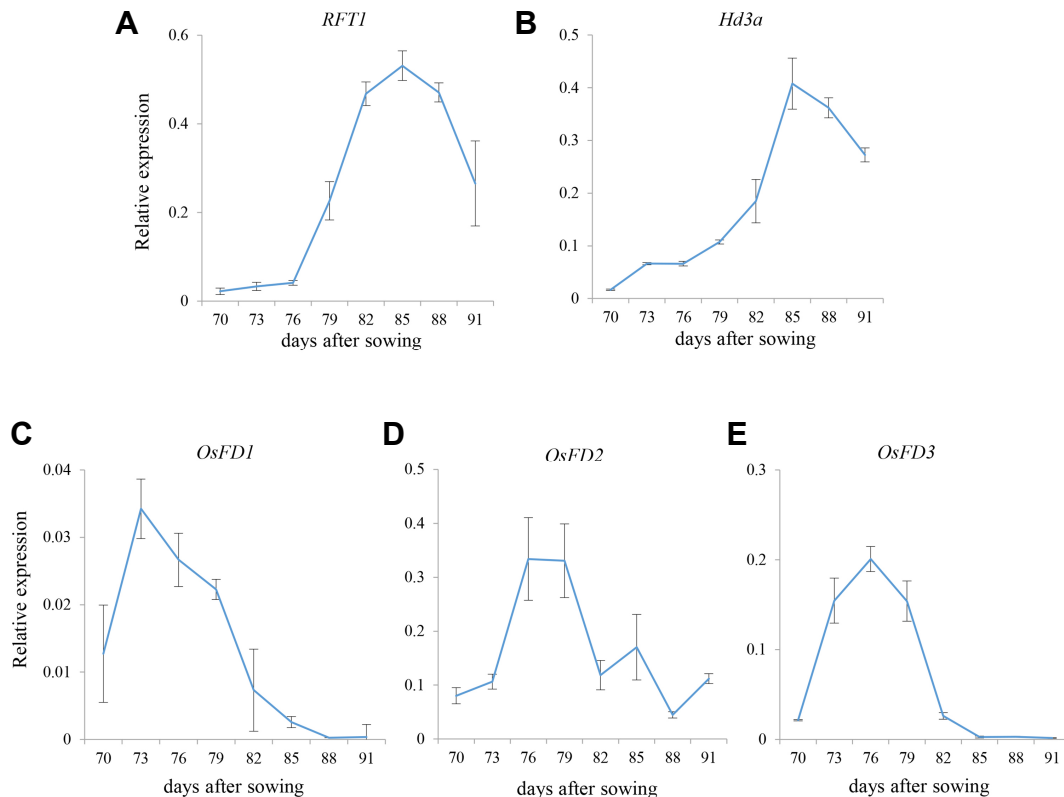
Analyses of three FUL-clade MADS box genes (*OsMADS14*, *OsMADS15*, and *OsMADS18*) showed that all were strongly expressed at Stage 2 in meristem tissues that had been induced by *RFT1* expression (Figs. 3G-3I). Expression of *OsMADS14* was very low at Stages 1 and 3 (Fig. 3G). Levels of *OsMADS15* transcripts were similar to those of *OsMADS14*, except that the former was expressed at a higher level than the latter in the floral organs at Stage 3 (Fig. 3H). Expression of *OsMADS18* was similar to *OsMADS15* but was detected at a moderate level at Stages 1 and 3 (Fig. 3I). Transcript levels for these three genes were low in WT samples at all three stages (Figs. 3G-3I). Our observations are consistent with the report that *OsMADS14* and *OsMADS15* expression is activated by *RFT1* in the SAM (Komiya et al., 2008; 2009).

Transcript of *OsMADS34* was present in Stage 2 *RFT1* samples, but not at Stages 1 and 3 (Fig. 3J). This was consistent with previous reports that the SEP-clade gene functions in inflorescence meristems (Kobayashi et al., 2012). However, another SEP gene, *OsMADS1*, was specifically expressed at Stage 1 in the greening tissues of *RFT1* plants (Fig. 3K). Transcripts of *OsMADS5* were present in all three stages (Fig. 3L), while *OsMADS7* transcripts were found mainly at Stages 1 and 2 (Fig. 3M), and *OsMADS8* transcripts at Stages 2 and 3 (Fig. 3N). Greening tissue-specific expression was also observed for *OsMADS50* when *RFT1* was over-expressed (Fig. 3O).

Three FD-like genes -- *OsFD1*, *OsFD2*, and *OsFD3* -- were expressed at all three stages in *RFT1*-expressing samples (Figs. 3P-3R), as was the *RFL* (Fig. 3S). In contrast, the floral organ identity genes *OsMADS2*, *OsMADS3*, and *OsMADS4* were expressed in the Stage 3 samples from *RFT1*-expressing calli, which confirming that the carpel- and stamen-like structures were indeed floral organs (Figs. 3T-3V).

#### Leaf-like organs covering inflorescence meristems are palea/lemma

Results from anatomical analyses suggested that the leaf-like organs developed from undifferentiated green calli of *RFT1*-transgenics resembled palea and lemma. We separated the outer leaf-like green organs from the non-green organs located inside (Figs. 4A-4F) and determined that the FUL genes, which are involved in inflorescence meristem development, were preferentially expressed in those inner organs at Stage 2 (Figs. 4G-4I). Another inflorescence marker, *OsMADS34*, was also strongly expressed in the inner organs, especially at Stage 2 (Fig. 4J). These results confirmed that the inner organs are inflorescence meristems. In contrast, *OsMADS1* was expressed at higher levels in the outer organs at both Stages 2 and 3 (Fig. 4K). Its relative expression was almost equivalent to that of the *Ubi1* control. *OsMADS1* was expressed at a lower level in the WT leaves and was further decreased in the *RFT1*-transgenic leaves (Fig. 1L).



**Fig. 5. Transcript levels of florigen and FD genes in leaf blades at different developmental stages.** Leaf blade samples were isolated at 3-d intervals starting from 70 DAS. Expression of *RFT1* (A), *Hd3a* (B), *OsFD1* (C), *OsFD2* (D), and *OsFD3* (E). Transcript levels are relative to *OsUbi1*. Error bars indicate standard deviation for 6 biological replicates.



This observation supported our proposal that the outer organs developed from *RFT1*-expressing calli were not leaves. Because the primary roles of *OsMADS1* are in regulating lemma/palea development (Jeon et al., 2000; Zhang et al., 2018), the outer organs were most likely palea/lemma in the spikelets. Expression of other SEP genes -- *OsMADS5*, *OsMADS7*, and *OsMADS8* - did not differ significantly between the inner and outer organs (Figs. 4L-4N), while that of *OsMADS50* was higher in the outer organs (Fig. 4O). As we had expected, the floral organ identity genes *OsMADS2*, *OsMADS3*, and *OsMADS4* were specifically expressed in the inner organs at Stage 3 (Figs. 4P-4R).

### Analysis of regulatory genes during plant development in the paddy

We investigated whether the differential expression patterns of the regulatory genes observed during inflorescence development from the calli are similar to those noted during normal meristem development in paddy-grown WT plants. Starting at 70 DAS, we collected leaf blades and shoot apices at 3-d intervals. Analyses of *RFT1* expression in the leaf samples showed low transcript levels until 76 DAS, which was followed by a rapid increase at 79 DAS, a peak at 85 DAS, and then a rapid decline (Fig. 5A). *Hd3a* expression presented a similar pattern, peaking at 85 DAS (Fig. 5B). These observations indicated that florigen molecules were starting to be produced at 79 DAS and reached a maximum level at 85 DAS.

We also studied expression levels of *OsFD1* in the leaves because that gene appears to form a complex with the florigen proteins and induces flowering by increasing the expression of *Ehd1*, *RFT1*, and *Hd3a* (Brambilla et al., 2017). Transcript levels of *OsFD1* were very low in the leaves at all stages, although they seemed to be slightly higher during the floral transition period when the florigen genes were actively expressed (Fig. 5C). In contrast, *OsFD2* and *OsFD3* were much more highly expressed, especially during those transitional stages (Figs. 5D and 5E).

We sampled vegetative SAMs and determined that Stage V1 occurred at 70 DAS while Stage V2 covered 73 to 76 DAS (Figs. 6A and 6B). Reproductive meristem regions were collected at R1, R2, R3, and R4 (Figs. 6D-6G). We also sampled panicles at In6, In7, and In8.

Transcript levels of *OsMADS14* were very low in the vegetative SAM before the transition, but they increased during that stage and rose to higher levels at R2 through R4 before declining to moderate levels during inflorescence stages 6 to 8 (Fig. 6H). In mature leaves, this MADS box gene was expressed at a very low level. Very similar patterns were observed for *OsMADS15* except that the gene was expressed at much lower levels in the vegetative SAMs (Fig. 6I). In contrast, *OsMADS18* was highly expressed at almost the same levels in the meristems at all developmental stages as well as in the mature leaves (Fig. 6J).

The expression pattern of *OsMADS34* was similar to that of *OsMADS15*, being highly expressed during the period of primary and secondary branch development (Fig. 6K). However, *OsMADS1*, *OsMADS5*, and *OsMADS8* were highly expressed at later stages, during inflorescence formation

(Figs. 6L, 6M and 6O), while *OsMADS7*, *OsMADS50*, and *RFL* did not show any specific patterns (Figs. 6N, 6P and 6S). As we had expected, the floral organ identity genes *OsMADS2* and *OsMADS4* were expressed strongly at the later stages of inflorescence development (Figs. 6Q and 6R). Similar to the pattern detected in the leaves, expression of *OsFD1* was much lower than that of *OsFD2* and *OsFD3* in the meristems. Transcript levels for *OsFD1* were higher during the transition and in the early reproductive stages but were low during inflorescence development (Fig. 6T). *OsFD2* and *OsFD3* were expressed during all stages, and at almost identical levels (Figs. 6U and 6V).

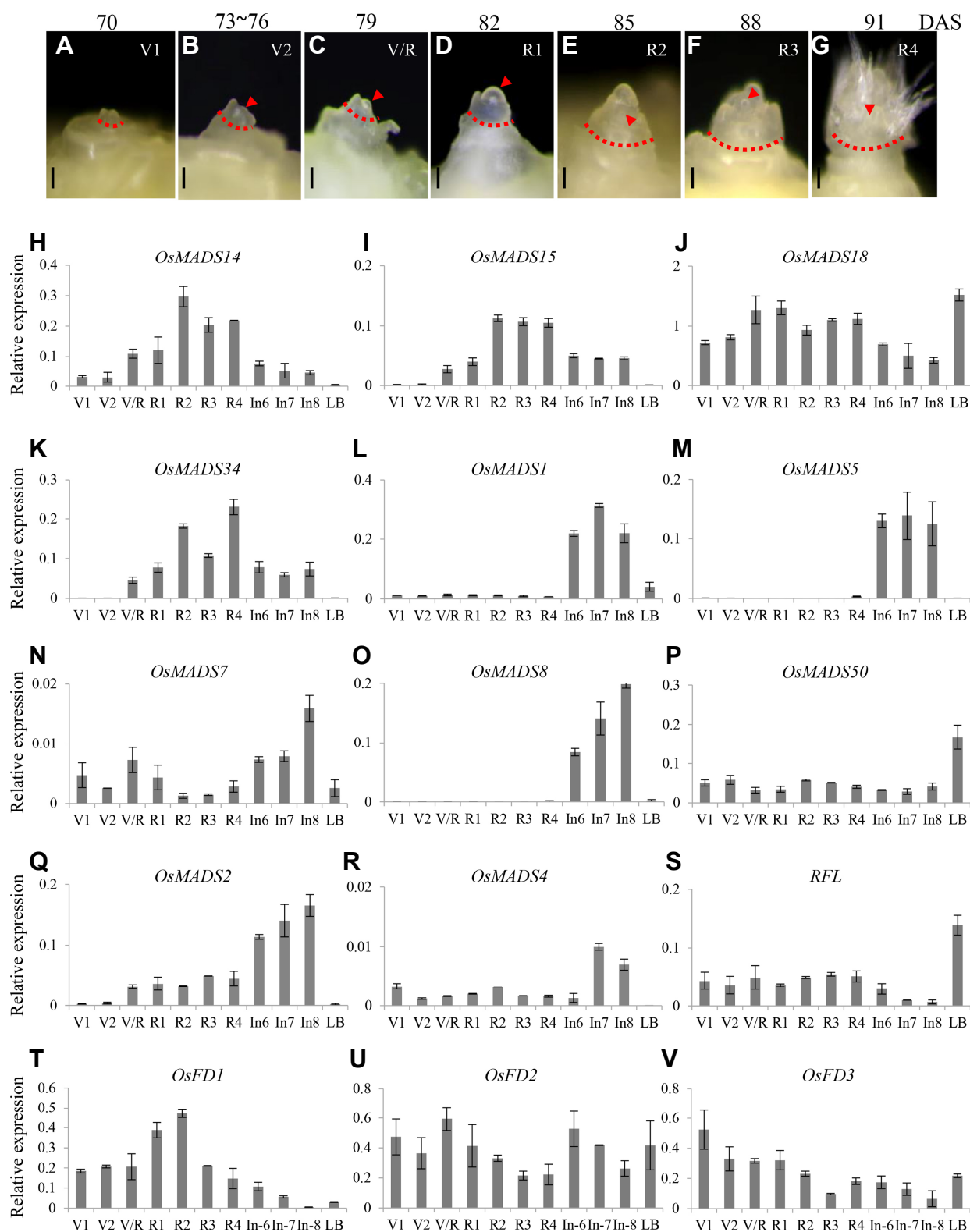
## DISCUSSION

### Overexpression of *RFT1* induces extremely early flowering

We demonstrated that strong expression of *RFT1* induced extremely early flowering in transgenic rice plants. A similar early flowering phenotype due to *Hd3a* overexpression has been reported (Izawa et al., 2002; Kobayashi et al., 2012; Kojima et al., 2002). Whereas most *Hd3a* transgenics produce plants that flower early and have only a few spikelets (Izawa et al., 2002; Kojima et al., 2002), we found here that a majority of our *RFT1*-transformed calli directly developed spikelets without the formation of any vegetative organs. Therefore, *RFT1* appears to be a stronger promoter of reproductive development. It will be needed to investigate how *RFT1* functions more strongly than *Hd3a*, especially because much of the study on floral transition in rice has been conducted with *Hd3a*. One possibility is that *RFT1* and *Hd3a* may form a FAC complex with different partner. Another possibility is that their direct targets may be different to each other.

Studies with *Arabidopsis*, rice, and *Triticum aestivum* have indicated that florigen proteins do not have activation functions, and formation of FAC with FD is needed to stimulate downstream expression of the target genes (Abe et al., 2005; Taoka et al., 2011; Wigge et al., 2005). Because overexpression of the florigen gene alone induces early flowering, one might think that other components in the FAC are not limiting factors during the transition to the reproductive stage. However, we observed that expression of *OsFD1* was highly induced in the *RFT1*-transgenic calli much before than *OsMADS14* and *OsMADS15* were induced (Fig. 3P), suggesting that *RFT1* induces *OsFD1* expression first in order to strongly promote target genes. This hypothesis is consistent with the report that *OsMADS14* and *OsMADS15* expression is strongly increased in the leaves of *OsFD1*-overexpression plants (Brambilla et al., 2017). It will be interesting to evaluate whether FAC directly enhances *OsFD1*. However, the latter was not enhanced in leaves from *RFT1*-overexpressing plants (Fig. 1R). Therefore, we propose that a repressor is possibly present in the leaf to suppress *OsFD1* expression. Alternatively, a positive element residing in reproductive tissues may be absent in the leaves.

Transient overexpression of *RFT1* and *Hd3a* downregulates endogenous *Ehd1*, *Hd3a*, and *RFT1* in the leaf (Brambilla et al., 2017). However, we did not observe such a



**Fig. 6. Expression analyses of regulatory genes in SAM of WT plants.** Shoot apex regions in paddy-grown WT plants, harvested at vegetative stages V1 (A) and V2 (B), transition stage (C), and reproductive stages R1 (D), R2 (E), R3 (F), and R4 (G). Scale bars: 200  $\mu$ m. Transcript levels of *OsMADS14* (H), *OsMADS15* (I), *OsMADS18* (J), *OsMADS34* (K), *OsMADS1* (L), *OsMADS5* (M), *OsMADS7* (N), *OsMADS8* (O), *OsMADS50* (P), *OsMADS2* (Q), *OsMADS4* (R), *RFL* (S), *OsFD1* (T), *OsFD2* (U), and *OsFD3* (V). Expression levels are relative to *OsUbi1*. Error bars indicate standard deviation for 6 biological replicates.

strong feedback suppression of endogenous *RFT1* and *Ehd1* in *RFT1*-overexpressing leaves, although *Hd3a* expression was somewhat reduced (Figs. 1U-1W). This discrepancy may be due to the nature of overexpression of the florigen genes. Whereas we constitutively expressed *RFT1*, Brambilla et al., (2017) transiently stimulated the gene.

### Overexpression of *RFT1* stimulates regulatory genes that are associated with reproductive development

The FUL-clade MADS box genes are major regulatory elements responsible for initiating reproductive development (Kobayashi et al., 2012). In rice, *OsMADS14*, *OsMADS15*, and *OsMADS18* function redundantly in that process. *OsMADS34* is also required for the transition (Kobayashi et al., 2012). When spikelets were developing directly from greening *RFT1* tissues, expression of those genes was strongly induced, preferentially in the reproductive meristematic tissues (Figs. 3G-3J). Therefore, our observations confirmed previous reports that these genes coordinately function in reproductive meristems.

The SEP-clade genes *OsMADS1*, *OsMADS5*, and *OsMADS7* primarily mediate floral organ development (Jeon et al., 2000; Kobayash et al., 2012). They are expressed later than the FUL-clade genes in developing inflorescences from paddy-grown plants. However, we noted here that the SEP genes were highly expressed in greening *RFT1* tissues before the spikelets formed (Figs. 3K-3M). This unexpected result suggested that the SEP genes have additional roles during the very early stages of reproductive development. Because they were induced before the FUL-clade genes, we must still examine whether the SEP-clade genes are also direct targets of RFT1-associate FAC. One possibility is that the SEP-clade genes are transiently required for the initiation of inflorescence development from greening tissues. *OsFD1* and related genes *OsFD2* and *OsFD3* could possibly be involved in their induction.

Whereas *LEAFY (LFY)* in Arabidopsis is expressed consistently in FM and functions primarily in specifying them by directly activating *AP1* (Weigel et al., 1992), the rice *LFY* homolog, *RFL*, is expressed mainly in incipient lateral branch primordia, where it is associated with maintenance of the inflorescence meristem (Rao et al., 2008). The rice protein suppresses the transition from inflorescence meristem to FM through interactions with APO1, the rice ortholog of *UNUSUAL FLORAL ORGANS* in Arabidopsis (Ikeda-Kawakatsu et al., 2012). We observed that expression levels of *RFL* were induced in the *RFT1*-overexpressing calli at all stages (Fig. 3S), thereby supporting its role during reproductive development.

*OsMADS50* is an ortholog of Arabidopsis *SOC1*, which is up-regulated in the meristem and stimulates early flowering (Lee et al., 2004; Ryu et al., 2009) This rice gene induces flowering preferentially under long-day conditions, but its role in the SAM during reproductive induction has been unknown. We observed that it was strongly induced in the *RFT1* greening tissues (Fig. 3O). During spikelet development, the gene was expressed preferentially in the palea/lemma tissues (Fig. 4K). These observations suggested that *OsMADS50* functions in green tissues during the formation of inflorescences. An interesting future study would

be to examine whether suppression of *OsMADS50* is necessary to develop those green tissues into reproductive tissues.

### *RFT1* overexpression does not immediately stimulate reproductive development

Although inflorescences developed directly from our transgenic calli, that process required more than one month in the shoot induction medium for such initiation. In addition, such reproductive development occurred only from the green tissues. This indicated that *RFT1* expression alone was insufficient. One possible explanation is that other components of the FAC were lacking in the undifferentiated calli, a hypothesis supported by our observation that *OsFD1* was induced in the green calli. Further investigation is needed on whether the FAC components *OsFD1* and *Gf14c* are expressed in undifferentiated tissues. In addition to *OsFD1*, *OsFD2* and *OsFD3* were also strongly induced in the *RFT1*-expressing tissues (Figs. 3P-3R). Therefore, it will be interesting to investigate whether RFT1 also forms a complex with OsFD2 and OsFD3. If they interact to each other, further study would be needed to evaluate whether the different complexes induce the same target genes or different downstream genes.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

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Supplementary Table S1. List of primers used in this study

**Primers for transgenic plants**

Primer name	Sequence (5'-3')
RFT1_F_HindIII	CGCAAGCTTATGGCCGGCAGCGGCAGGGA
RFT1_R_SpeI	GCACTAGTCTAGGGGTAGACCCTCCTGC

**Primers for qRT-PCR**

Ubi1_F	CACGGTTCAACAACATCCAG
Ubi1_R	TGAAGACCCTGACTGGGAAG
Hd3a_F	AGCCCAAGTGACCCTAACCT
Hd3a_R	GTTGTAGAGCTCGGCGAAGT
RFT1_F	TTCTGGTACCACTGGAGCA
RFT1_R	GTCTCAGCTTAGCTATAGCT
OsMADS1_F	ATCACCATCAGGGTCTTCTC
OsMADS1_R	CAACCATGTCTGCTGCTTCA
OsMADS2_F	GAACACGAGAACAAGCTGCT
OsMADS2_R	TTCAATCCAGTGGTGGATCA
OsMADS3_F	CAGCGTGATCAACCTGAAGA
OsMADS3_R	CAACACACACAATATGGCAC
OsMADS4_F	CAATCTGCGGGACAAGATG
OsMADS4_R	GAGCTCCAGCTCCCTTATGC
OsMADS5_F	GCTTCATATATCTTGCCAAG
OsMADS5_R	TTGGTTGAGGTGATCCATGT
OsMADS7_F	TGGGTTCTTCCATCCACTTG
OsMADS7_R	CGTCATCATCATGGTAGCCA
OsMADS8_F	CACCTTGCAGATCGGGTTTA
OsMADS8_R	ATCTGTGTCGTCACATCCGT
OsMADS14_F	TGAGCGCTACTCCTATGCAG
OsMADS14_R	GCCTTCAGTTTCCTATATTCGTG
OsMADS15_F	TGAGTCCATTTCCGAGCTG
OsMADS15_R	TCTTCTGCCTCTCCACCAGT
OsMADS18_F	ATAACAGTCAAAGCCAACCA
OsMADS18_R	CAATATCGCTGGAAGATGGA
OsMADS34_F	AGCAGCTCCACTGGCTACAAATGA
OsMADS34_R	AGGTCGCAGAGTTCATCAAG
OsMADS50_F	AAAGCTGACGCTGATGGTTTG
OsMADS50_R	TTTACCGAGATCCAGCTTATTCCTGG
OsFD1_F	TTCAGGTGGACGACCTTAGC
OsFD1_R	GCTAGCAGCTGCCAACACT
OsFD2_F	AGGTCGACCAAAAACAAGC
OsFD2_R	TCCTCTCATGCCTGAC
OsFD3_F	CAGAGTTCCCACCGAAGAAG
OsFD3_R	AATAATGGTCCCCAATCCAA
RFL_F	GTTGTCCGAGGAGCATGAC
RFL_R	CCTCGCCGGGCTCCGTCACC