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DEFORMED FLORAL ORGAN1 (*DFO1*) regulates floral organ identity by epigenetically repressing the expression of *OsMADS58* in rice (*Oryza sativa*)

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Summary

- Floral organ identity in plants is controlled by floral homeotic A/B/C/D/E-class genes. In *Arabidopsis thaliana,* several epigenetic repressors that regulate these floral organ identity genes have been characterized. However, the roles of epigenetic factors in rice floral development have not been explored in detail. Here, we report the identification and functional characterization of a rice epigenetic repressor, *DEFORMED FLORAL ORGAN1* (*DFO1*) gene, which causes abnormal floral morphology when mutated.
- We isolated *dfo1* by mapping, and confirmed its function by rescue experiments, combined with genetic, cytological and molecular biological analysis.
- We showed that *DFO1* is constitutively expressed and encodes a nuclear-localized protein. Mutation of *DFO1* causes the ectopic expression of C-class genes in the *dfo1-1* mutant, and overexpression of *OsMADS58*, a C-class gene, phenocopies the *dfo1* mutants. *In vitro* and *in vivo* experiments demonstrated that DFO1 interacts with the rice polycomb group (PcG) proteins (OsMSI1 and OsiEZ1). Remarkably, trimethylation of histone H3 lysine 27, a mark of epigenetic repression, is significantly reduced on *OsMADS58* chromatin in the *dfo1-1* mutant.
- Our results suggest that *DFO1* functions in maintaining rice floral organ identity by cooperating with PcG proteins to regulate the H3K27me3-mediated epigenetic repression on *OsMADS58*.

Introduction

Flowers display diversity in their morphology. A typical dicot flower consists of four successive whorls of organs: sepals, petals, stamens and pistils. The ABCDE model based on studies from dicot species, such as *Arabidopsis thaliana* and *Antirrhinum majus*, has been widely accepted. In this model, working from the outside to the center of the flower, A (*AP1*/*AP2*)- and E (*SEP1/2/3*)-class genes specify sepal identity in whorl 1; A-, B (*AP3*/PI)- and E-class genes regulate petal formation in whorl 2; B-, C (*AG*)- and E-class genes coordinately determine stamen identity in whorl 3; C- and E-class genes specify pistils in whorl 4; and C- and D (*STK*)-class genes are redundantly involved in ovule development. Most of these genes encode MADS-box transcription factors and have been shown to regulate homeotic transformations of floral organs (Coen & Meyerowitz, 1991; Pelaz *et al.*, 2000; Theissen & Saedler, 2001; Pinyopich *et al.*, 2003; Ditta *et al.*, 2004).

The grass family (Poaceae) contains many important crops, including rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and maize (*Zea mays*). Among them, rice has been selected as the model plant for functional genomics studies of crops, and its spikelet morphogenesis is important to rice yield. Rice flowers are distinct from those of dicots as they consist of two pairs of sterile glumes (rudimentary glumes and empty glumes) and one floret, including a lemma and a palea, two lodicules, six stamens and a pistil (Bommert *et al.*, 2005; Itoh *et al.*, 2005). The two lodicules corresponding to the dicot petals are considered to be whorl 2, the six stamens are thought to be whorl 3 and the pistil is thought to be whorl 4, whereas the lemma and palea are assumed to be whorl 1, but the question of what is equivalent to the dicot sepal remains elusive (Nagasawa *et al.*, 2003; Prasad *et al.*, 2005; Yadav *et al.*, 2007; Yao *et al.*, 2008; Yoshida & Nagato, 2011; Sang *et al.*, 2012).

Accumulating evidence has shown that the ABCDE model is at least partially applicable to rice floral development. Five types of MADS-box genes are partially conserved in rice and have been

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shown to be involved in the specification of rice floral development. *OsMADS14*, *OsMADS15*, *OsMADS18* and *OsMADS20* are considered to be A-class (*AP1*/*Ful*-like) genes, and their functions are largely unknown, except that *OsMADS15* is required for the regulation of palea size (Wang *et al.*, 2010). *OsMADS2*, *OsMADS4* and *OsMADS16* are orthologs of the *Arabidopsis* B-class genes (*PI* and *AP3*, respectively), and have been shown to regulate the development of lodicules and stamens (Nagasawa *et al.*, 2003; Xiao *et al.*, 2003; Whipple *et al.*, 2004; Yao *et al.*, 2008). *OsMADS3* and *OsMADS58* are thought to be homologs of the *Arabidopsis* C-class gene *AG* (Kramer *et al.*, 2004; Yamaguchi *et al.*, 2006). *OsMADS3* is thought to play a key role in the specification of stamen identity, late anther development and floral meristem determinacy, whereas *OsMADS58* is crucial for the specification of floral meristem determinacy and pistil architecture (Yamaguchi *et al.*, 2006; Hu *et al.*, 2011). Pistil identity in rice has also been shown to be determined by *DROOPING LEAF* (*DL*), a YABBY family gene (Nagasawa *et al.*, 2003; Yamaguchi *et al.*, 2004). Rice contains two STK-like genes considered to be homologous to D-class genes in *Arabidopsis*: *OsMADS13* and *OsMADS21*. *OsMADS13* plays a key role in the specification of ovule identity and floral meristem determination, whereas *OsMADS21* has lost its function during evolution (Dreni *et al.*, 2007, 2011; Dreni & Kater, 2014). Moreover, *OsMADS1* (*LHS1*), *OsMADS5*, *OsMADS6*, *OsMADS7*, *OsMADS8* and *OsMADS34* are considered to be E-class genes that specify the identities of four whorl floral organs (Jeon *et al.*, 2000; Gao *et al.*, 2010; Wang *et al.*, 2010; Li *et al.*, 2011a).

Studies from *Arabidopsis* have suggested that polycomb group (PcG) proteins serve as another group of crucial regulators in plant floral organ development, and further studies have demonstrated that PcG proteins function in floral development by repressing the expression of the floral identity genes by trimethylation of H3 lysine 27 (H3K27me3) (Goodrich *et al.*, 1997; Hennig *et al.*, 2003; Katz *et al.*, 2004; Calonje *et al.*, 2008; Kim *et al.*, 2012). The PcG proteins have at least two forms in *Drosophila*, including polycomb repressive complexes 1 (PRC1) and 2 (PRC2). Among them, PRC2 plays an essential role in inhibiting the initiation of transcription by altering chromatin structure, whereas PRC1 is responsible for maintaining the stability of this inhibited chromatin structure (Otte & Kwaks, 2003; Lund & van Lohuizen, 2004; Pien & Grossniklaus, 2007). In *Drosophila*, PRC2 is composed of four parts: Enhancer of Zeste (E(z)), Suppressor of Zeste (12) (Su(z)12), Extra Sex Combs (ESC) and p55 (Wang *et al.*, 2006; Margueron & Reinberg, 2011), whereas PRC1 consists of the proteins Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and dRING1 (Levine *et al.*, 2004). Components of PRC2 are well conserved between animals and plants, but PRC1 components are only partially conserved in plants and animals (Luo *et al.*, 1999; Ohad *et al.*, 1999; Brock & Van Lohuizen, 2001; Pien & Grossniklaus, 2007; Bratzel *et al.*, 2010). In *Arabidopsis*, PRC2 has at least three forms, FIS2-PRC2, EMF2-PRC2 and VRN2-PRC2, and each contains the ESC homolog (FIE) and homologs of E(z) (MEA, CLF or SWN), Su(z)12 (EMF2, FIS2 or VRN2) and a homolog of p55 (MSI1) (Ahmad *et al.*, 2010). In rice, several PcG-like proteins have been found: OsiEZ1 and OsCLF (E(z)), OsFIE1 and OsFIE2 (ESC) as well as OsEMF2a and OsEMF2b (Su(z) 12) (Hennig & Derkacheva, 2009; Luo *et al.*, 2009). Among them, only OsFIE1, OsFIE2 and OsEMF2b have been genetically identified and characterized (Yang *et al.*, 2012; Zhang *et al.*, 2012; Nallamilli *et al.*, 2013).

In *Arabidopsis*, the RING-finger proteins AtRING1A/1B and AtBMI1A/B, together with chromodomain protein LHP1 and the plant-specific protein EMF1, compose the plant PRC1-like complex (Zheng & Chen, 2011). Of these, EMF1 together with EMF2-PRC2 performs an essential role in the PcG-mediated floral repression mechanism (Calonje *et al.*, 2008; Kim *et al.*, 2012). Despite these significant advances, our understanding of the molecular mechanism of PcG-mediated floral repression in monocot species still remains limited and fragmented.

In this study, we isolated two allelic mutants of the *DEFORMED FLORAL ORGAN1* (*DFO1*) gene (*dfo1-1* and *dfo1-2*) that exhibit abnormal floral morphology with changed floral organ number and identity. We showed that *DFO1* encodes a nuclear-localized protein, which is orthologous to *Arabidopsis thaliana* EMBRYONIC FLOWER1 (EMF1). Further analyses revealed that *DFO1* is constitutively expressed in rice and its mutation causes ectopic expression of C-class genes in the *dfo1-1* mutant. Remarkably, we also found that overexpression of the C-class gene *OsMADS58* causes partially similar phenotypic defects to the *dfo1* mutants. Finally, we showed that DFO1 physically interacts with rice PcG proteins OsMSI1 and OsiEZ1, and chromatin immunoprecipitation analysis revealed significant reduction of H3k27me3 levels on *OsMADS58* chromatin. These results suggest that DFO1 is involved in the repression of *OsMADS58* expression in an H3K27me3-mediated manner. Together, our results illustrate the pathway of EMF1/DFO1-mediated floral repression by epigenetic regulation of its targets in floral development.

Materials and Methods

Plant materials

Two allelic mutants (*dfo1-1* and *dfo1-2*) were isolated from an M_2 population of the ^{60}Co-irradiated variety 93-11 (*O. sativa* L., *indica*). Genetic analysis demonstrated that the mutant trait was controlled by a single recessive gene (Supporting Information Table S1). The F_2 mapping population was generated from a cross between *dfo1-1* and 02428 (*O. sativa* L., *japonica*). Plants were grown in paddy fields during the natural growing season at Nanjing Agricultural University, Nanjing, China.

Microscopy

For paraffin sectioning, *dfo1-1* and wild-type flowers at various stages were fixed in formalin–acetic acid–alcohol (FAA) solution, and samples were treated as described by Xiao *et al.* (2009). Microtome sections (8 μm thick) were stained with 0.05% toluidine blue and observed with a ECLIPS E80i light microscope (Nikon, Tokyo, Japan). For scanning electron microscopy

(SEM), flowers from different developmental stages were fixed in 2.5% glutaraldehyde solution overnight at 4°C, and then prepared as described by Zhou *et al.* (2011).

Gene mapping and rescue test of the *dfo1-1* mutant

Ten plants with *dfo1* phenotypic defects were first identified from the F_2 mapping population of *dfo1* and 02428 for preliminary mapping using 220 genome-wide simple sequence repeat (SSR) markers; then, 1058 F_2 recessive plants were used for fine mapping with genetic markers designed by comparison of the local genomic sequences of 93-11 and Nipponbare (*O. sativa*, *japonica*) obtained from the National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/nuccore/AP008207 (CM000126)] (Table S2).

To test whether *LOC_Os01g12890* was responsible for *dfo1*, the *DFO1* cDNA was cloned into the pCUbi1390 binary vector (*Ubi-DFO1*) (primer sequences are listed in Table S3), which was then introduced into *Agrobacterium tumefaciens* strain EHA105 and used to infect calli prepared from *dfo1*/02428 F_3 recessive homozygous seeds (*d-5*). The transgenic plants were grown in a glasshouse to observe the phenotypes.

Real-time PCR analysis

Total RNA was isolated using the RNA prep pure plant kit (TIANGEN, Beijing, China). The first-strand cDNA was synthesized using Oligo (dT) 18 as primer, and PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China) for reverse transcription. Rice *ubiquitin* (*UBQ*) was used as endogenous control. Real-time PCR analysis was performed using an ABI 7500 Fast real-time PCR system with the SYBR Green Mix (Bio-Rad, Hercules, CA, USA) and three biological repeats. Primers used for real time-PCR analysis are listed in Table S4.

Pro_{DFO1}-*GUS* reporter gene construction and analysis

A 1.7-kb promoter fragment upstream of the *DFO1* ATG start codon was fused to the *β-glucuronidase* (*GUS*) reporter gene and cloned into pCAMBIA1381Z (primer sequences are listed in Table S3). The Pro_{DFO1}-*GUS* vector was transformed into *japonica* var Nipponbare by the *Agrobacterium*-mediated method (Hiei & Komari, 2008). GUS staining was performed as described by Zhou *et al.* (2011).

RNA *in situ* hybridization

Wild-type and *dfo1-1* spikelets of different stages were fixed in FAA (RNase-free) fixative solution at 4°C overnight, and then dehydrated through an ethanol series (50%, 70%, 80%, 95% and 100%) and xylene, embedded in paraffin (Paraplast Plus; Thermo Fisher, Waltham, MA, USA) and sectioned at 8 μm using a Leica RM2235 microtome (Leica, Wetzlar, Germany). The construct of the *OsMADS58* probe was generated as described by Yamaguchi *et al.* (2006) (primer sequences are listed in Table S3). RNA *in situ* hybridization was performed as described previously (Bradley *et al.*, 1993). Images were obtained using a Nikon ECLIPSE80i microscope and photographed using a Nikon CD5Ri1P camera.

Yeast assay

The MATCHMAKER pGBKT7/ADT7 Two-Hybrid system (Clontech Laboratories, Mountain View, CA, USA) was used to test transcriptional activation ability. Various fragments were cloned into pGBKT7 and pGADT7 to construct BD-DFO1/Nt/M1/M2/Ct/iEZ1/OsMSI1 and AD-Ct, respectively (primer sequences are listed in Table S3). All constructs were transformed into the recipient strain AH109 and selected on SD/–Trp–Leu plates at 30°C for 3–5 d. The activation ability was assayed on selective medium [SD/-Trp-Leu-His-Ade (QDO) plus X-α-gal] plates, and interactions were assayed on selective medium [SD/-Trp-Leu-His-Ade (QDO)] plates.

Pull-down (*in vitro*) assays

Four fragments of DFO1 (Nt, M1, M2, Ct) were cloned into the pMAL-C2x vector (NEB, Hitchin, Hertfordshire, UK) to construct the fusion proteins MBP-Nt/M1/M2/Ct, and OsMSI1 was cloned into the pET-30 (a) vector to form the His-OsMSI1 fusion protein (primer sequences are listed in Table S3). The pull-down analyses were performed as described by Lin *et al.* (2012). The western blots were detected with anti-maltose-binding protein (anti-MBP) (NEB) or anti-His (Millipore, Billerica, MA, USA) antibodies at 1 : 1000 dilutions, and the second antibody was anti-rabbit or anti-mouse (1 : 5000 dilutions; Abmart, Shanghai, China). The western blots were detected using enhanced chemiluminescence (ECL) reagent (Bio-Rad, Hercules, CA, USA).

Bimolecular fluorescence complementation (BiFC) assay

The full-length cDNA of *DFO1* was cloned into the p2YN (eYFP) vector to construct the DFO1-eYFPN fusion protein. OsMSI1 and OsIEZ1 were cloned into the p2YC (eYFP) vector to produce OsMSI1-eYFPC and OsiEZ1-eYFPC fusion proteins, respectively (primer sequences are listed in Table S3). The BiFC analyses were performed in tobacco, as described previously (Ren *et al.*, 2014). The mCherry ER-rk CD3-959 was used as ER (endoplasmic reticulum) maker (Nelson *et al.*, 2007).

Subcellular localization of DFO1 protein

To explore the subcellular localization of DFO1, the *DFO1* cDNA and three fragments (1–328, 1–410 and 1–562) were fused with green fluorescent protein (GFP) and inserted in the pA7-GFP vector between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator (primer sequences are listed in Table S3). The *35S-DFO1-GFP* plasmid was transformed into rice protoplasts and onion epidermal cells according to the protocols described by Xiao *et al.* (2009). pA7-GFP alone was employed as the control, and we used the mCherry-tagged rice prolamin box binding factor

(RPBF-mCherry) vector as a nuclear marker (Kawakatsu *et al.*, 2009).

Construction of the *pUbi-OsMADS58* vector

An 819-bp cDNA fragment of the full-length *OsMADS58* gene was amplified from rice var 93-11 cDNA using the primers listed in Table S3. The full-length cDNA was cloned into the pCUBi1390 vector, and the construct *Ubi-OsMADS58* was transformed into *A. tumefaciens* strain EHA105.

Chromatin immunoprecipitation (ChIP) analysis

ChIP assays were performed as described previously (Zhang *et al.*, 2012). Anti-trimethyl-Histone H3 (Lys27) was purchased from Millipore (#CS200603; http://www.millipore.com). Primer sequences are listed in Table S5. All assays were performed at least three times from two biological replicates.

Results

Isolation and characterization of the *dfo1* mutants

To investigate the regulation of rice floral development, we identified two mutants with similar phenotypic defects in floral morphology, and named them *deformed floral organ 1-1* (*dfo1-1*) and *dfo1-2*, respectively (Fig. S1). In this report, we focus on our further studies on *dfo1-1*. The *dfo1-1* mutant resembled the wild-type in the vegetative stage, and its flowering time was not obviously changed (Fig. S2a), but its height exhibited a smaller stature compared with that of the wild-type (Figs 1a,d, S2b; Table S6). In addition to plant architecture, another obvious defect was the morphology of the *dfo1-1* spikelet, which displayed severe defects in the development of the palea (Fig. 1b,e). Notably, most spikelets in *dfo1-1* were sterile as they lacked stamens, whereas only a few had normal pollen, and the seeds were deformed because of the shrunken palea (Fig. 1c,f).

The *dfo1-1* mutant shows defects in palea identity

In a wild-type rice floret, a lemma and a palea form an interlocked structure outside the whole floret (Fig. 2a,s). The palea in the wild-type consists of two parts: the body of the palea (bop) and two marginal regions of the palea (mrps) (Fig. 2b,c). By contrast, in the *dfo1-1* mutant, the lemma and palea exhibited abnormal shapes and lost the interlocked lemma/palea structure (Fig. 2d). Notably, the *dfo1-1* palea was much smaller and incurved, and the mrp of *dfo1-1* paleas was replaced by green tissues, and some stigmatic papillae-like tissues were observed on the tip of the mrp (Fig. 2e–g). In addition, some lemmas of the *dfo1-1* spikelet grew a long awn (Figs 1f, 2d). SEM analysis revealed that the mrp of the wild-type palea had a smooth surface (Fig. 2h–m), whereas the identity of the mrp in *dfo1-1* had a rough surface similar to that of the wild-type carpel with stigmatic-like tissues at the tip (Fig. 2n–r). Moreover, the bop of the *dfo1-1* palea had more bract hairs and the size of the bop was reduced (Fig. 2n). Anatomical observation of the *dfo1-1* palea revealed that its inner epidermal cells were smaller than those of the wild-type, and the number of vascular bundles in *dfo1-1* paleas resembled that in the lemmas (Fig. 2s,t). As the bop was shrunken and the mrp was replaced by a pistil-like mrp (plm), the spikelet formed an open hull instead of a hooked-hull (Figs 2s–u, 3i).

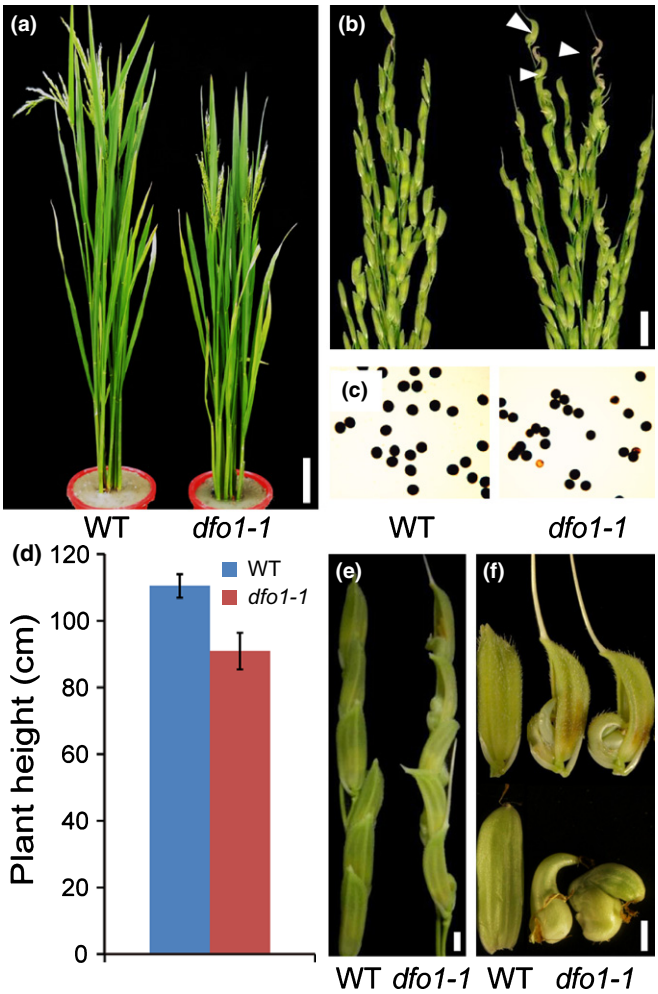

Fig. 1 Morphological analyses of rice (*Oryza sativa*) wild-type (WT) and *dfo1-1* mutant. (a) Phenotypes of wild-type and *dfo1-1* plants at the heading stage. (b, e) Panicles of wild-type and *dfo1-1* plants at the heading stage. Arrowheads indicate the abnormal spikelets. (c) KI-I_2 staining of wild-type and *dfo1-1* pollen. (d) Heights of wild-type and *dfo1-1* plants. Error bars, ± SD ($n = 20$). (f) Shapes of wild-type and *dfo1-1* seeds. Bars: (a) 10 cm; (b) 1 cm; (c, e, f) 2 mm.

These morphological characteristics of the palea suggest that the bop of the *dfo1-1* palea has acquired partial lemma-like identity, and the mrp has acquired pistil-like identity. Taken together, we conclude that *DFO1* is involved in the specification of palea identity.

Inner floral organs display defects in identity in the *dfo1-1* mutant

The *dfo1-1* inner floral organs also exhibited severe morphological abnormalities (Fig. 3). The wild-type rice floret

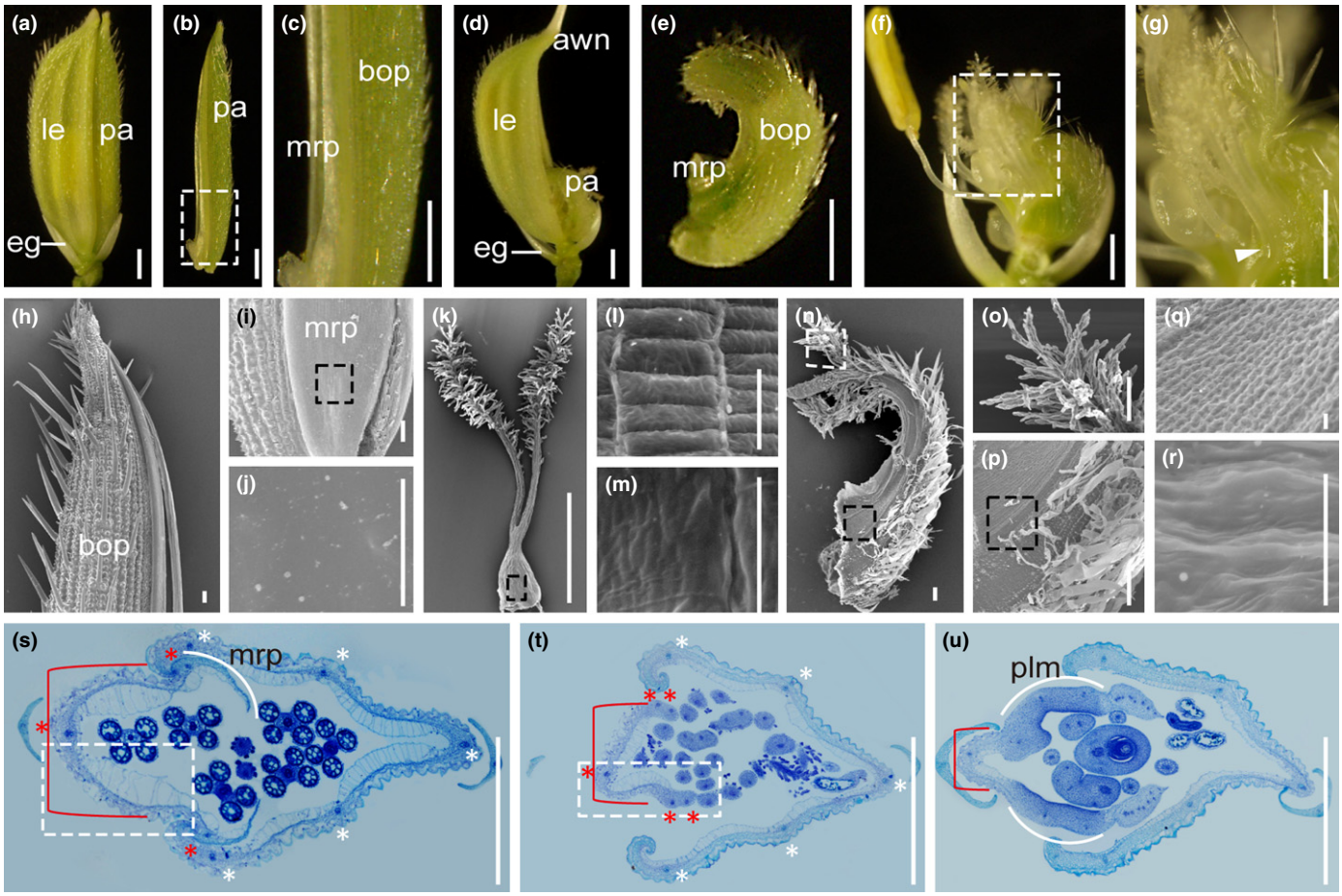

Fig. 2 The rice (*Oryza sativa*) *dfo1-1* mutant shows defects in palea identity. (a) Wild-type spikelet with two empty glumes, one lemma and one palea in the outer whorl. (b) The palea of the wild-type. (c) Magnification of the dashed box in (b). (d, e) *dfo1-1* spikelet with an incurved palea. (f) *dfo1-1* palea with stigmatic papillae-like tissues on the tip of the marginal region of the palea. (g) Close-up of the white dashed box in (f). Arrowhead indicates the pistil-like organs. (h) Scanning electron microscopy observation of wild-type palea. (i) mrp of wild-type palea. (j) Close-up of mrp of wild-type palea in black dashed box of (i). (k) Pistil of wild-type spikelet. (l) Wild-type ovarian epidermal cells in black dashed box of (k). (m) Close-up of wild-type ovarian epidermal cells in (l). (n) Appearance of *dfo1-1* paleas. (o, p) Magnifications of the white and black dashed boxes in (n), respectively. (q) Magnification of the black dashed box in (p). (r) Close-up of (q). (s–u) Cross-sections of wild-type (s) and *dfo1-1* (t, u) spikelets. White and red asterisks indicate vascular bundles of lemma and palea, respectively. White dashed boxes in (s, t) indicate the inner epidermal cells of the wild-type and *dfo1-1* paleas. Red brackets in (s–u) indicate the bop of the wild-type and *dfo1-1* palea. White curves in (s, u) indicate the mrp of the wild-type and *dfo1-1* palea. bop, body of palea; eg, empty glume; le, lemma; mrp, marginal region of the palea; pa, palea; plm, pistil-like mrp. Bars: (a–g, k, s–u) 1 mm; (h, i, n–p) 100 μm; (j, l, m, q, r) 10 μm.

consisted of two lodicules in whorl 2, six stamens in whorl 3 and one pistil in whorl 4 (Fig. 3a,g). However, in the *dfo1-1* mutant, the second whorl, the lodicules, showed a partial loss of identity and some assumed the identity of a pistil (Fig. 3b,c, i; Table 1). In the third whorl, the number of stamens per floret was reduced (Table 1); some stamens resembled the wild-type, whereas others were homeotically transformed into pistil-like organs (plos), and yet others were transformed into chimeras that were part pistil and part stamen (Fig. 3d,e). Remarkably, in some *dfo1-1* florets, all of the inner floral organs were transformed into stigmatic papillae-like tissues, and also had more than one pistil (Fig. 3f,h; Table 1). By contrast, in whorl 4, the pistil grew normally in the *dfo1-1* mutant. These morphological observations suggest that the lodicules and stamens in the *dfo1-1* mutant have acquired the identity of the pistil, and that *DFO1* is required for specification of the identities of these floral organs.

Abnormal early floral organ development in the *dfo1-1* mutant

To further examine the early developmental defects in the *dfo1-1* mutant, we analyzed the early spikelets of the wild-type and *dfo1-1* mutant using SEM. At spikelet stage 4 (Sp4, formation of the palea primordium), wild-type spikelets initiated palea primordia, whereas the *dfo1-1* mutant did not (Ikeda *et al.*, 2004; Fig. 4a,f). At stage Sp6 (formation of the stamen primordia), palea primordia had formed and the stamen primordia had initiated in the wild-type, whereas the development of the palea and stamen primordias in *dfo1-1* spikelets was dramatically delayed (Fig. 4b,g). Later, in Sp6, unlike the wild-type (Fig. 4c), the number of stamen primordia in *dfo1-1* spikelets decreased and some primordia seemed to have lost their identities (Fig. 4c,h). After the Sp7 stage (formation of the pistil primordium), the pistil primordia formed in the center of the six stamens in the wild-type; the *dfo1-1*

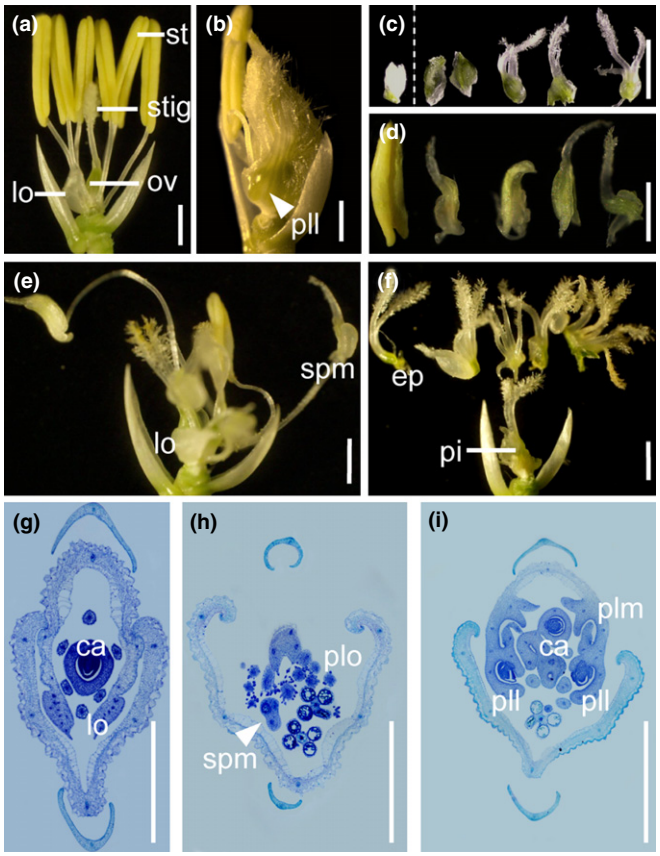

Fig. 3 Inner floral organs of rice (*Oryza sativa*) *dfo1-1* mutant. (a) Wild-type spikelet comprising two lodicules, six stamens and one pistil. (b) *dfo1-1* spikelet lodicule with pistil-like features. (c) Wild-type lodicule (left) and pistil-like lodicules in *dfo1-1* spikelets (right). (d) Stamen morphologies in *dfo1-1* spikelets. (e) Partial stamens homeotically transformed into stamen–pistil mosaic organs in a *dfo1-1* spikelet. (f) A *dfo1-1* spikelet in which all stamens were converted into stigmatic papillae-like tissues and an extra pistil was found in the inner whorl. (g–i) Transverse sections of wild-type (g) and *dfo1-1* (h, i) spikelets. (h) Several stigmatic papillae-like tissues found in a *dfo1-1* spikelet. (i) Two pistils ectopically growing at the position of the lodicules. ca, carpel; ep, extra pistil; lo, lodicule; ov, ovule; pi, pistil; pll, pistil-like lodicule; plm, pistil-like marginal region of the palea (mrp); plo, pistil-like organ; spm, stamen–pistil mosaic organ; st, stamen; stig, stigma. Bars, 1 mm.

spikelets formed normal pistil primordia similar to the wild-type, but the number of stamens was decreased (Fig. 4d,i), Finally, at stage Sp8 (formation of ovules and pollen), the wild-type formed a normal spikelet, whereas the *dfo1-1* spikelet formed a long awn on the tip of the lemma and its paleas were incurved and smaller than those of the wild-type (Fig. 4e,j). These results suggest that the palea and stamen identities were altered in early floral development of the *dfo1-1* mutant.

DFO1 is the rice *EMF1* gene

To determine the molecular function of *DFO1*, we isolated the gene by map-based cloning. Ten individuals showing the *dfo1* phenotype were chosen from the F_2 progeny of a cross between *dfo1* and 02428, and the mutated locus was preliminarily mapped to a region between the SSR markers RM272 and RM5496 on the short arm of chromosome 1. Fine mapping with 1058 recessive plants using eight primers further mapped the *dfo1* locus to a 60-kb region (Fig. 5a) (primer sequences are listed in Table S2), in which 14 open reading frames (ORFs) were predicted by the Rice GAAS (rice genome automated annotation system, http://ricegaas.dna.affrc.go.jp; Table S7). Sequence analysis showed that the second ORF (*LOC_Os01g12890*), a putative ortholog of *Arabidopsis EMF1*, differed between the wild-type and both mutants. The *LOC_Os01g12890* gene contains four exons and three introns, and encodes a 116.4-kDa protein with 1057 amino acids (Fig. 5b). We found that, in *dfo1-1*, a T was changed to an A, resulting in a premature stop, whereas, in *dfo1-2*, a G was deleted, causing a frame shift and premature stop in the last exon (Fig. 5b). To confirm these mutations, we amplified the genomic fragment spanning mutated sites using dCAPS (derived cleaved amplified polymorphic sequence) markers (primer sequences are listed in Table S2), and digested these products with the restriction enzymes *Hind*III and *Afl*II; the results confirmed that these mutations existed in the two mutants (Fig. 5c).

To test whether *LOC_Os01g12890* was responsible for *dfo1*, the *DFO1* coding region driven by the *UBQ* promoter was transformed into calli derived from *dfo1* homozygous seeds. Twenty-five transgenic lines were obtained, among which 13 lines were resistant to hygromycin and harbored the transgene, as well as displaying the wild-type phenotypes (Fig. 5d). Taken together, these results confirmed that *LOC_Os01g12890* (rice *EMF1*) was the *DFO1* gene.

Constitutive expression of *DFO1*

We examined the spatial and temporal expression of *DFO1* to study how it functioned at the molecular level. Real-time PCR analyses detected *DFO1* transcripts in all organs, including young roots, stems, leaves and panicles. Notably, *DFO1* was more highly expressed in leaves and young panicles relative to

Table 1 Numbers of floral organs in rice (*Oryza sativa*) wild-type and *dfo1-1* plants

	Abnormal palea ratio (%)	Lodicule	Stamens							Pistil–stamen chimeras				Ovules			Stigmas	
			6	5	4	3	2	1	0	3	2	1	0	3	2	1	>2	2
WT (50)	0	2 (100%)	50	0	0	0	0	0	0	0	0	0	50	0	0	50	0	50
dfo1-1 (100)	88.7	2 (31%)	9	11	6	8	10	6	50	3	8	24	65	4	16	80	90	10

A total of 50 wild-type and 100 *dfo1-1* spikelets were examined.

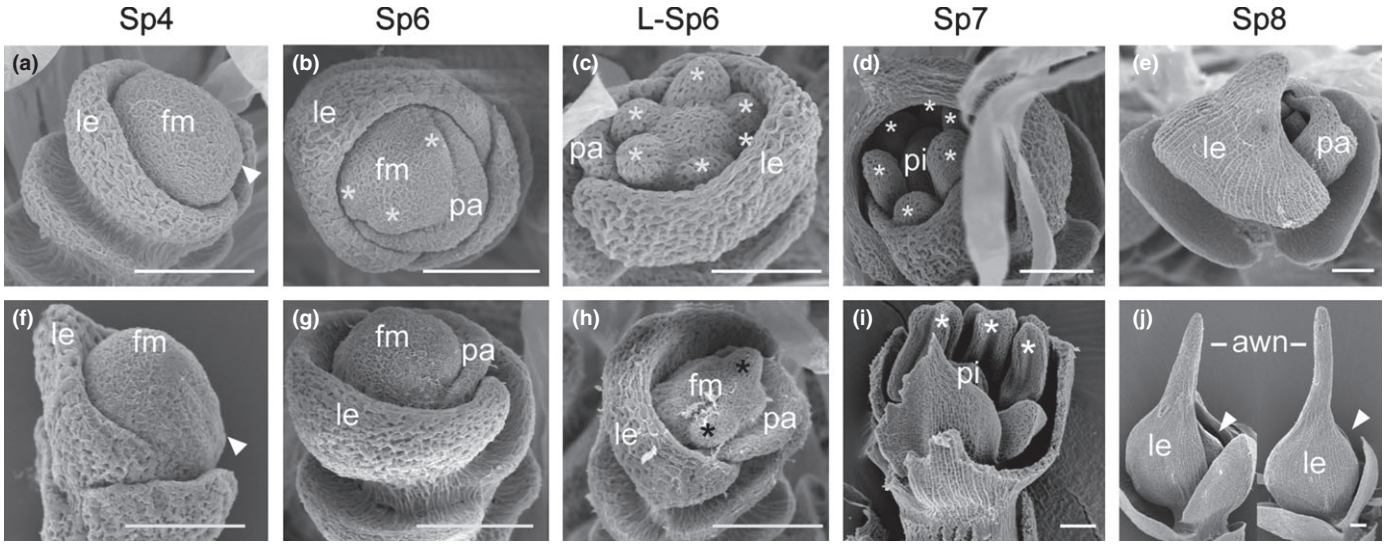

Fig. 4 Scanning electron micrographs of rice (*Oryza sativa*) wild-type and *dfo1-1* spikelets at early stages. (a–e) Wild-type spikelets at different stages. (a) Sp4. (b) Sp6. (c) Later in Sp6. (d) Sp7. (e) Sp8. (f-j) *Dfo1-1* spikelets at stages Sp4 (f), Sp6 (g), later in Sp6 (h), Sp7 (i) and Sp8 (j). Arrowheads in (a, f) indicate the palea primordia and in (j) indicate the incurved paleas. White and black asterisks indicate the stamens in wild-type and *dfo1-1* spikelets, respectively. fm, floral meristem; le, lemma; pi, pistil; pa, palea. Bars, 50 μm.

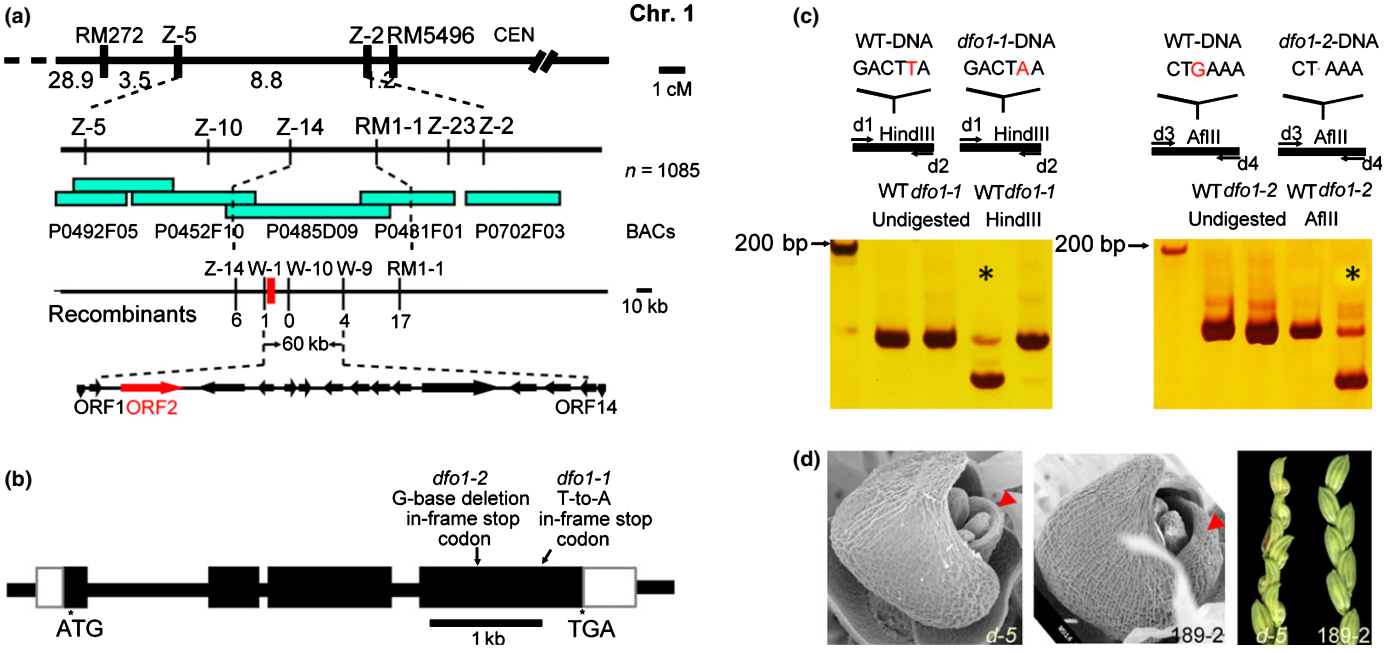

Fig. 5 Map-based cloning of DEFORMED FLORAL ORGAN1 (*DFO1*). (a) Fine mapping of the *DFO1* gene on chromosome 1. The *DFO1* locus was mapped to a 60-kb region that contained 14 predicted open reading frames (ORFs). (b) Structure of the *DFO1* gene and the mutations in the *dfo1-1* and *dfo1-2* alleles. *dfo1-1* changed a single T to A; *dfo1-2* deleted a single G. Lines indicate introns and black boxes indicate exons. (c) Molecular identification of the positions of the two mutations by dCAPS (derived cleaved amplified polymorphic sequence) markers. The left and right panels show the dCAPS analysis associated with the *dfo1-1* and *dfo1-2* alleles, respectively. Black stars represent the digested fragments. (d) Characterization of T_0 transgenic plants. The left panel shows the scanning electron microscopy (SEM) analysis of recessive F_3 plant (*d-5*) and T_0 complemented plant (189-2) spikelets (red arrowheads indicate the paleas). The right panel shows the floral phenotypes of mature panicles of *d-5* and 189-2 plants.

other organs (Fig. 6a). To precisely detect the spatial and temporal expression patterns of *DFO1*, we constructed a *DFO1* promoter-GUS plasmid (*pDFO1-GUS*) and transformed it into Nipponbare calli (an *O. sativa*, *japonica* cultivar). Consistent with the real-time PCR results, GUS activity was predominantly detected in young roots, stems, sheaths, leaves, panicles and all floral organs (Fig. 6b). At the reproductive stage, GUS was highly expressed in young panicles (Fig. 6b, v), and weakly in mature panicles (Fig. 6b, vii). GUS expression was also observed in lodicules, stamens and pistils (Fig. 6b, viii). Together, these results indicate that *DFO1* is constitutively expressed.

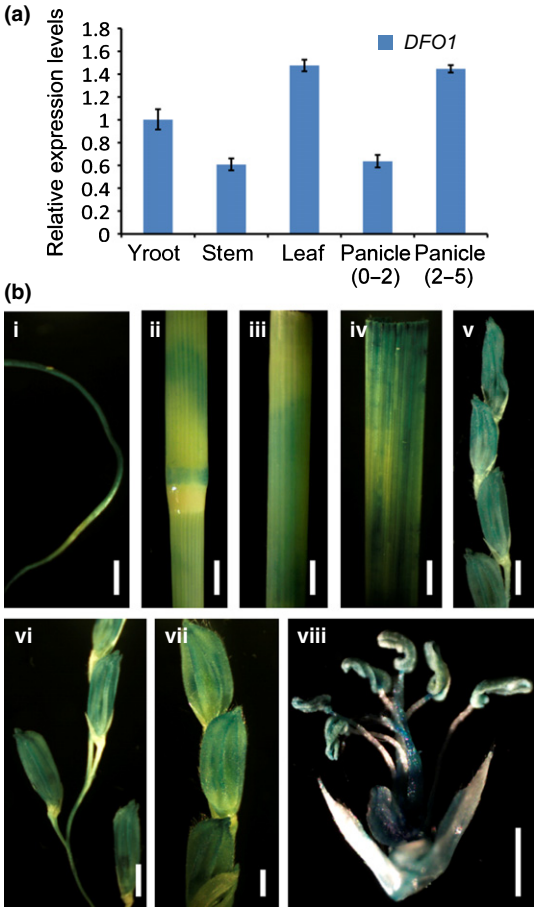

Fig. 6 Analysis of the expression of the *DEFORMED FLORAL ORGAN1* (*DFO1*) gene in rice (*Oryza sativa*). (a) Real-time PCR analyses showing that *DFO1* is expressed constitutively in the young root (yroot), stem, leaf and panicles (0–2 and 2–5 cm). (b) β-Glucuronidase (GUS) staining of various tissues and developmental stages of spikelets in the Pro_{DFO1}:GUS transgenic line. Left to right (i–viii) are root, stem, sheath, leaf, panicle (< 5 cm), panicle (5–10 cm), panicle (flowering time) and a spikelet, respectively. *Ubiquitin* (*UBQ*) was used as an internal control in the real-time PCR analyses. Error bars, ± SD ($n = 3$). Bars, 2 mm.

DFO1 encodes a nuclear protein

Phylogenetic analysis found that DFO1 belongs to the EMF family of transcription factors, but is located in a small clade distinct from EMF1, a plant-specific transcription factor which is crucial to *Arabidopsis* vegetative development and shares 20% amino acid identity with DFO1 (Aubert *et al.*, 2001; Kim *et al.*, 2012) (Figs 7a, S3, S4). In addition, several domains were identified in the DFO1 polypeptide, including four potential nuclear localization signals (NLSs), a putative ATP/GTP-binding motif (P-loop) and an LXXLL motif (where L is leucine and X is any amino acid) (Figs 7c, S4, S5a). The LXXLL motif has been demonstrated to mediate interactions that can activate or repress transcription (Heery *et al.*, 1997; Calonje *et al.*, 2008). Moreover, homology analysis found several regions at the N- and C-terminus that were conserved in EMF1 proteins of *Arabidopsis* and several monocots (Figs S3, S4).

To determine the subcellular localization of the DFO1 protein, a plasmid encoding full-length DFO1 protein fused to the N-terminus of GFP was constructed and transiently expressed in onion epidermal cells. The results showed that the DFO1-GFP fusion protein was localized in the nucleus (Fig. 7b). In order to determine whether the putative NLSs were essential for the nuclear localization of DFO1, we constructed and transformed three plasmids ($DFO1_{1–328}$-GFP, $DFO1_{1–410}$-GFP and $DFO1_{1–562}$-GFP) into rice seedling stem protoplasts. $DFO1_{1–410}$-GFP and $DFO1_{1–562}$-GFP showed the typical nuclear localization pattern, whereas $DFO1_{1–328}$-GFP was dispersed in the cytoplasm (Fig. S5b). We therefore concluded that amino acids 328–410 of DFO1 are necessary for its nuclear localization.

We next analyzed the transcriptional activity of DFO1 using a yeast two-hybrid system. Several fragments encoding the conserved regions were fused with the DNA-binding domain of the yeast GAL4 protein (GAL4-BD) to assay their transcriptional activities in yeast (Fig. 7c). No transcriptional activation was observed with the full-length, Nt, M1 and Ct fragments of DFO1, whereas yeast cells transformed with the M2 fragment grew well on the SD/–Leu–Trp–His–Ade–x–α-gal plate (Fig. 7c). Further, we found that the Ct fragment can repress the transcriptional activity of the M2 fragment (Fig. 7c). This may have occurred because the Ct fragment contains an LXXLL motif which is a likely suppressor of transcriptional activation (Li *et al.*, 2011b).

Based on these results, we suggest that DFO1 encodes a nuclear protein that may act as a transcriptional regulator.

Altered expression of floral organ identity genes in *dfo1-1*

The *dfo1-1* mutant displayed significantly altered spikelet morphology with conversion of floral organ identity and number, suggesting that the expression of some floral organ identity genes was altered in floral development. Real-time PCR analyses found that the A-class (*OsMADS14*) and B-class (*OsMADS2* and *OsMADS16*) genes were down-regulated in *dfo1-1* inflorescences (20 mm), and the C-class (*OsMADS3*, *OsMADS58* and *DL*) and D-class (*OsMADS13*) genes were up-regulated, whereas the E-class (*OsMADS1*) and A-class (*OsMADS15*) genes did not vary (Fig. 8a,b).

To further confirm the *dfo1-1* floral organ identities, we analyzed the expression of the A-class genes (*OsMADS14* and *OsMADS15*) and C-class genes (*OsMADS3*, *OsMADS58* and *DL*) in the lemmas and paleas of the wild-type and *dfo1-1*, respectively. We found that *OsMADS15* was down-regulated in *dfo1-1* lemmas and paleas, whereas *OsMADS14* was only decreased in *dfo1-1* paleas (Fig. 8c). All of the C-class genes were ectopically expressed in both *dfo1-1* lemmas and paleas (Fig. 8d). In *dfo1-1* stamen–pistil chimeric organs, *OsMADS16* and *OsMADS3*, which regulate stamen identity in the wild-type, were decreased. *OsMADS58* and *DL*, as well as D-class (*OsMADS13*) genes, which are known to be involved in the regulation of pistil identity, were also ectopically expressed in *dfo1-1* stamen–pistil chimeras (Fig. 8e).

These changes in expression further suggest that the paleas acquired pistil-like and lemma-like identities and the stamens acquired pistil-like identities in *dfo1-1* spikelets.

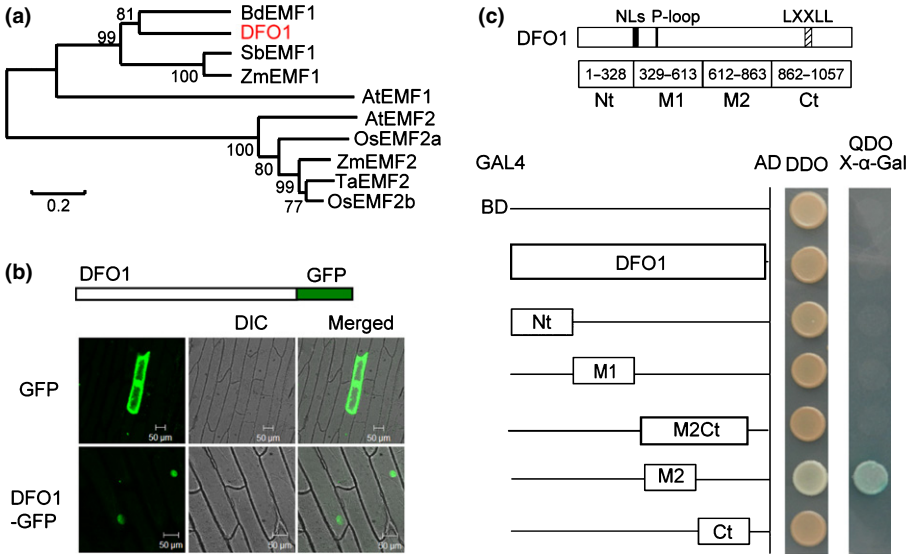

Fig. 7 Molecular identification of DEFORMED FLORAL ORGAN1 (DFO1). (a) Phylogenetic tree of the *EMBRYONIC FLOWER1* (*EMF*) genes in *Arabidopsis*, rice and other monocots. The phylogenetic tree was constructed using MEGA version 5.0. At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*; Ta, *Triticum aestivum*; Zm, *Zea mays*. (b) Subcellular localization of the DFO1-GFP fusion protein in onion epidermal cells. An onion epidermal cell expressing free green fluorescent protein (GFP) showed fluorescence in the nucleus and the cytoplasm (i–iii). Onion epidermal cells expressing DFO1-GFP solely showed fluorescence in the nucleus (iv–vi). DIC, Differential Interference Contrast. (c) Transcriptional activation assays in yeast. Schematic representation of DFO1 (top). Thick black boxes, nuclear localization signals (NLSs); thin black boxes, GTP-ATP-binding motif (P-loop motif); hatched boxes, LXXLL motif. Various DFO1 fragments were designed based on regions conserved in DFO1 and its homologs (middle). Transcriptional activation assays used the yeast two-hybrid system (bottom). DDO, control medium (SD/-Trp-Leu); QDO, selective medium (SD/-Trp-Leu-His-Ade).

Overexpression of *OsMADS58* results in phenotypes similar to those of *dfo1*

Previous studies have shown that *AG* is the direct target of *EMF1* in *Arabidopsis*, and *EMF1* can repress *AG* expression (Calonje *et al.*, 2008). In rice, the homologs of *AG* are *OsMADS3* and *OsMADS58*, and ectopic expression of *OsMADS3* led to the homeotic transformation of lodicules into stamens, but no morphological alterations were observed in lemmas and paleas (Kyozuka & Shimamoto, 2002). However, the development of paleas was affected in *dfo1* mutants. We further examined the expression pattern of *OsMADS58* in the *dfo1-1* mutant by RNA *in situ* hybridization. As reported previously (Yamaguchi *et al.*, 2006), early expression of *OsMADS58* was detected in the wild-type floral meristem at stage Sp5 (Fig. 9a), and was obviously detectable in the stamens and carpel primordia during the development of these floral organs (Sp6–Sp8) (Fig. 9b–d). Notably, *OsMADS58* transcripts were detected in the lemma and palea primordia, as well as floral meristem, at stage Sp5 in the *dfo1-1* mutant (Fig. 9e). Moreover, strong expression of *OsMADS58* was subsequently observed in whorl 1 to whorl 3 of *dfo1-1* florets (Sp6–Sp7) (Fig. 9f–h). Thus, we hypothesized that the ectopic expression of *OsMADS58* in the *dfo1-1* mutant probably affected the development of the palea.

To test our hypothesis, we overexpressed *OsMADS58* in Nipponbare under the control of the maize *UBQ* promoter, and generated 11 independent transgenic rice lines. All transgenic lines that ectopically expressed the *OsMADS58* gene displayed phenotypes partially similar to those of the *dfo1* mutants (Fig. 9i). In these transgenic lines, the palea was incurved and reduced (Fig. 9j), the spikelets had two or three pistils, and some pistils developed at the position of mrp, whereas the stamen morphology did not change (Fig. 9k,l). Moreover, in these transgenic lines, expression of *OsMADS58* was greatly elevated, whereas that of the A-class genes (*OsMADS14* and *OsMADS15*) was obviously reduced relative to the wild-type (Nipponbare) (Fig. 9m). These results suggest that ectopic expression of *OsMADS58* represses the expression of A-class genes, and support the hypothesis that the phenotypes of the *dfo1-1* mutant are partially caused by the resulting ectopic expression of the C-class floral regulator gene *OsMADS58*.

DFO1 interacts with putative rice PcG proteins and mediates the enrichment of H3K27me3 on *OsMADS58* chromatin

In *Arabidopsis*, EMF1 has been reported to interact with the EMF2-PRC2 component MSI1 and functions as a PcG protein that mediates floral repression (Cao *et al.*, 2002; Calonje *et al.*, 2008; Kim *et al.*, 2012). Therefore, we hypothesized that DFO1 might interact with OsMSI1, which shares high homology with MSI1. To test this hypothesis, we performed a yeast two-hybrid assay, and the results showed that the Ct fragment of DFO1 interacted with OsMSI1 (Fig. 10a). We also tested possible interactions with other rice putative PRC2 components, including OsCLF, OsiEZ1, OsEMF2a, OsEMF2b, OsFIE1 and OsFIE2. We found that the Ct fragment of DFO1 also interacted with OsiEZ1 in yeast (Fig. 10a), but the others did not (data

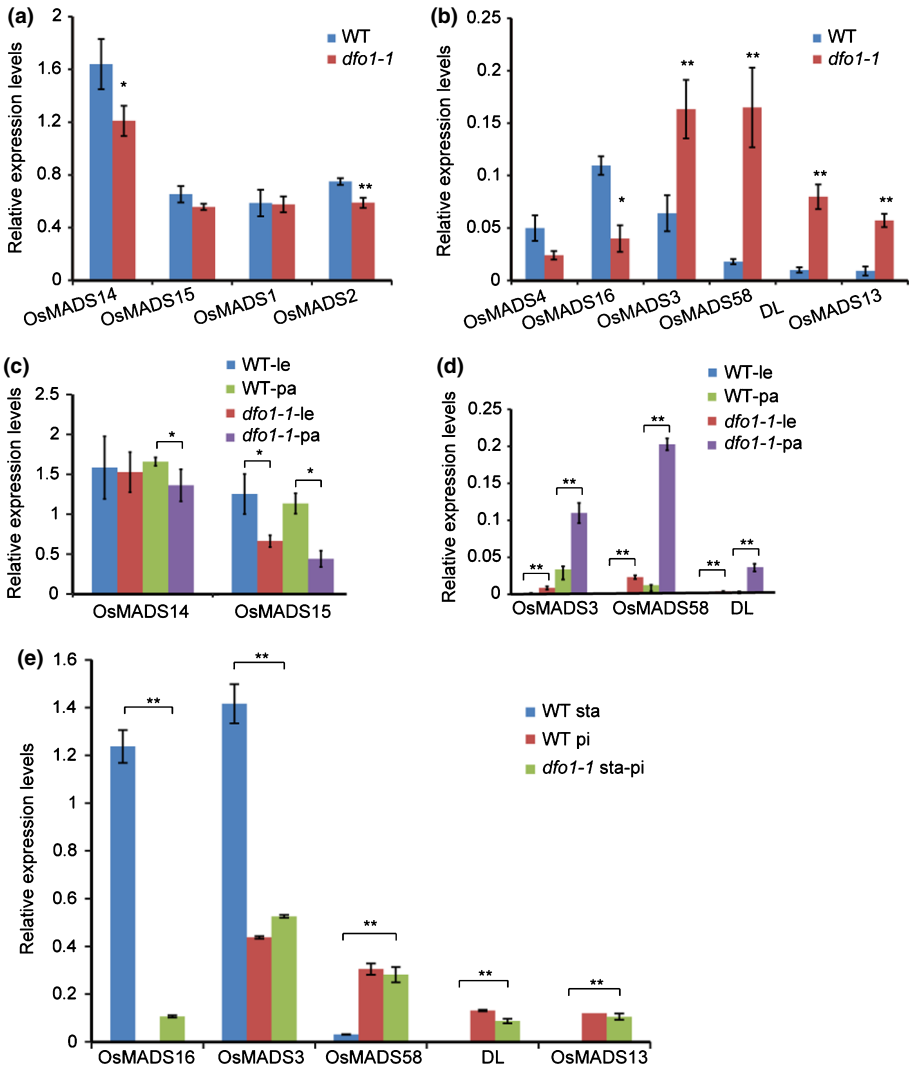

Fig. 8 Real-time PCR analyses of the expression of rice (*Oryza sativa*) floral organ identity genes in wild-type and *dfo1-1* floral organs. (a, b) Relative expression levels of floral organ identity genes in wild-type and *dfo1-1* young inflorescences (20 mm). (c, d) Expression of A-class (*OsMADS14* and *OsMADS15*) (c) and C-class (*OsMADS3*, *OsMADS58* and *DL*) (d) genes in the lemmas and paleas of wild-type and *dfo1-1* spikelets. (e) Expression of B-, C- and D-class genes in wild-type stamens, pistils and *dfo1-1* stamen–pistil chimeras. The *ubiquitin* (*UBQ*) gene was used as an internal control. Error bars, ± SD ($n = 3$). Student's *t*-test was used for statistical analysis (*, $P \leq 0.05$; **, $P \leq 0.01$). le, lemmas; pa, paleas; pi, pistils; sta, stamens; sta-pi, stamen–pistil chimeras.

not shown). Pull-down experiments showed that the Ct fragment of DFO1 bound OsMS11 *in vitro* (Fig. 10b). Finally, our BiFC assays demonstrated these interactions in *Nicotiana benthamiana* leaf cells, which showed that DFO1 interacted with OsMSI1 and OsiEZ1 proteins in the nucleus (Fig. 10c). These results support the hypothesis that DFO1 participates in PcG-mediated gene repression.

The PRC2 complex suppresses its targets by adding H3K27me3 marks, and we found that DFO1 interacted with the core components of the rice PRC2 complex. Therefore, we hypothesized that the H3K27me3 modification was altered on DFO1 targets. In *dfo1* mutants, *OsMADS58* was dramatically ectopically expressed in leaves (Fig. 10e). We next examined H3K27me3 modification of *OsMADS58* chromatin by ChIP-real-time PCR in wild-type and *dfo1-1* leaves, and found that H3K27me3 was significantly enriched on the promoter and translational start regions of *OsMADS58* chromatin in the wild-type, but to a lesser extent in the *dfo1-1* mutant (Fig. 10f). These results suggest that DFO1 mediates the H3K27me3 modification on *OsMADS58* chromatin.

Discussion

DFO1 is involved in the specification of palea identity

The lemma and palea are grass-specific organs, which develop an interlocked structure and specify seed production. Our results show that *DFO1* is a regulator of palea identity. The *osmads15* mutant and plants in which *OsMADS15* was down-regulated by RNA interference showed severe defects in their paleas (Wang *et al.*, 2010). The defects in paleas are the most obvious phenotypes of *dfo1* mutants, as 88.7% of the *dfo1-1* spikelets showed deformed paleas (Table 1) and the expression of *OsMADS15* was decreased in *dfo1* paleas. Overexpression of *OsMADS58* led to decreased expression of *OsMADS15* and the transformation of mrps into plos. These results suggest that *DFO1* may be a factor specifying the fate of paleas through the regulation of *OsMADS58* and *OsMADS15* during floral development.

In grass flowers, the lemma is generally considered to be a bract and the palea has often been proposed to be homologous to the

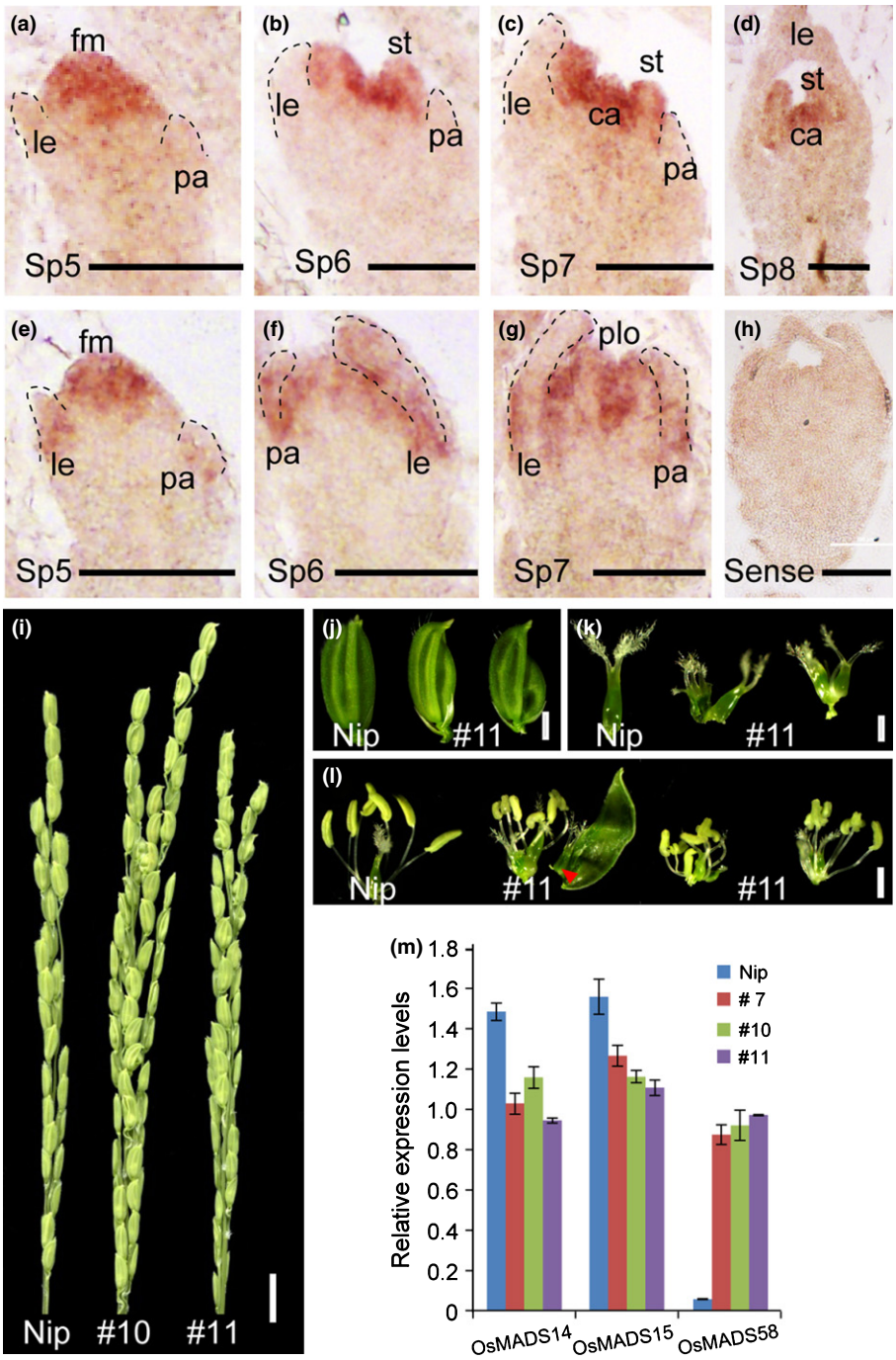

Fig. 9 *In situ* localization of *OsMADS58* in wild-type and *dfo1-1* flowers and effects of ectopic expression of *OsMADS58* in rice (*Oryza sativa*). (a–d) *In situ* localization of *OsMADS58* transcripts in wild-type flowers at stages Sp5 (a), Sp6 (b), Sp7 (c) and Sp8 (d). (e–g) *In situ* localization of *OsMADS58* transcripts in *dfo1-1* flowers at stages Sp5 (e), Sp6 (f) and Sp7 (g). (h) The sense probe of *OsMADS58* was used as a negative control. (i–k) Spikelet phenotypes in Pro_{Ubi}::*OsMADS58* transgenic lines. (i) Panicles of Nipponbare (Nip) and transgenic lines. (j) T_0 transgenic line spikelets with incurved and shrunken paleas. (k) Increased numbers of pistils in line #11. (l) The number of stamens in line #11 is unchanged, but ectopic pistil-like organs are present on the marginal region of the palea (mrp) (red arrowhead) and interior of the spikelets. (m) Expression of floral identity genes in Nip and Pro_{Ubi}::*OsMADS58* lines. The *ubiquitin* (*UBQ*) gene was used as an internal control. Error bars, ± SD ($n = 3$). ca, carpel; fm, floral meristem; le, lemma; pa, palea; plo, pistil-like organ; st, stamen. Bars: (a–h) 100 μm; (i–l) 2 mm.

prophyll (Kellogg, 2001; Ohmori *et al.*, 2009). Although the lemma and palea display similar cellular characteristics, the lemma is larger than the palea, and has five vascular bundles, whereas the palea has only three. The palea has been shown to consist of the bop and two mrps (Fig. 3) (Nagasawa *et al.*, 2003; Prasad *et al.*, 2005; Yuan *et al.*, 2009). Previous studies have indicated that the lemma and palea are the first whorl organs of grass florets (Bowman, 1997; Xiao *et al.*, 2003; Preston *et al.*, 2009). However, together with other studies, we hold that the lemma is not equivalent to the palea as the first whorl of a rice flower, and the mrp is the homolog of the dicot sepal, whereas the bop is not (Nagasawa *et al.*, 2003; Prasad *et al.*, 2005; Yadav *et al.*, 2007; Yao *et al.*, 2008; Sang *et al.*, 2012). This notion is supported by the following observations. (1) In the *dfo1-1* mutant, the palea had several defects: the bop of the palea was incurved and shrunken, and the mrp was converted to an ovary-like structure and also showed stigmatic papillae-like tissues at the tip. However, the lemma largely retained its identity (Fig. 2). In addition, some mutants (*osmads6*, *mfs1*, *dp1*, *rep1* and *mfs1*) also showed defects in the palea, but not in the lemma (Ohmori *et al.*, 2009; Jin *et al.*, 2011; Sang *et al.*, 2012; Ren *et al.*, 2013). Moreover, in the *osmads15* mutant, the bop of the palea was shrunken, whereas

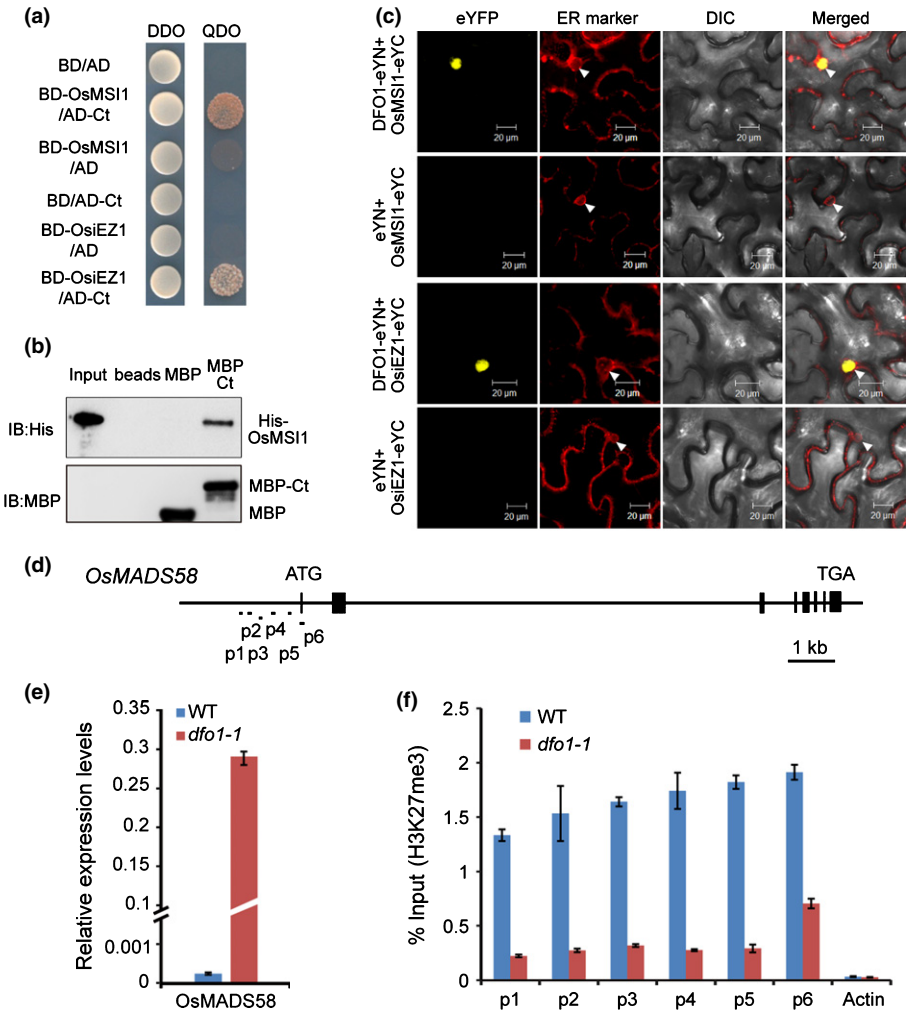

Fig. 10 Interactions between DEFORMED FLORAL ORGAN1 (DFO1) and putative rice (*Oryza sativa*) polycomb group (PcG) proteins, and trimethylation of lysine 27 of histone H3 (H3K27me3) analysis of *OsMADS58* chromatin. (a) Yeast two-hybrid assay showing that DFO1 interacts with OsMSI1 and OsiEZ1. DDO, control medium (SD/-Trp-Leu); QDO, selective medium (SD/-Trp-Leu-His-Ade). (b) MBP-Ct and His-OsMSI1 interact *in vitro*, but not MBP itself. (c) Bimolecular fluorescence complementation (BiFC) assays showing that DFO1 can interact with OsMSI1 and OsiEZ1 in the nuclei of leaf cells of *Nicotiana benthamiana*. The signals of enhanced yellow fluorescent protein (eYFP) were not detected in the corresponding negative controls. White arrowheads indicate the nuclear membrane. DIC, Differential Interference Contrast; ER, ER maker (mCherry ER-rk CD3-959). (d) Genomic structure of *OsMADS58*, and the regions tested in chromatin immunoprecipitation (ChIP assays. (e) Expression of *OsMADS58* in wild-type and *dfo1-1* leaves. (f) Trimethylation of lysine 27 of histone H3 (H3K27me3) analysis of *OsMADS58* chromatin. Error bars, ± SD ($n = 3$). Bars, 20 μm.

the mrps of the palea largely retained their identity, indicating that the bop and mrps have different origins (Wang *et al.*, 2010). (2) In *Arabidopsis*, the sepals are considered to be the first whorl, and ectopic expression of *AG* leads to stigmatic papillae at the sepal tips, and a few ovules often develop on the sepals (Mizukami & Ma, 1992). In our studies, the florets of *Ubi-OsMADS58* transgenic lines displayed incurved and reduced paleas, and ectopic pistils grew at the position of the mrp, whereas the lemmas showed no changes. (3) In the rice *osmads3 osmads58* double mutant, the carpel is replaced by a palea-like organ (Dreni *et al.*, 2011), and the B-class gene mutant *spw1* and OsMADS2 + OsMADS4 double RNAi plants showed transformation of lodicules into mrp-like organs (Yao *et al.*, 2008).

DFO1 negatively regulates C-class genes

Mutations in *DFO1* led to the ectopic expression of *OsMADS58* and, in transgenic *Ubi-OsMADS58* flowers, multiple pistils were found and some pistils grew ectopically at the position of the mrp. These phenotypes were partially similar to those of *dfo1* mutants, indicating that they functioned in the same pathway, and that *DFO1* might negatively regulate *OsMADS58*, because *OsMADS58* was not expressed in the lemmas, paleas or leaves of the wild-type, whereas its expression was dramatically increased in these organs in the *dfo1-1* mutant. Another C-class gene, *OsMADS3*, was weakly ectopically expressed in the *dfo1-1* mutant. This increased expression of *OsMADS3* could cause the observed changes in the lodicules of *dfo1* spikelets, because, in transgenic *35S::OsMADS3* lines, the lodicules were homeotically transformed into stamens (Kyozuka & Shimamoto, 2002). Similarly, in the *dfo1-1* mutant, *c.* 69% of spikelets lost their lodicules, which were replaced with plos (Fig. 4; Table 1). Therefore, *DFO1* may play a key role in the maintenance of floral organ identity by repressing the transcription of *OsMADS3* and *OsMADS58* in the lodicules and palea.

The *DL* gene, which specifies pistil and lemma identities, was ectopically expressed in *dfo1-1* mutant lemmas and paleas, as well as in pistil–stamen mosaic organs. Recently, the *DL* gene has been considered to be a factor in awn development, and further studies have found that decreasing the expression of *DL* affects awn formation (Toriba & Hirano, 2014). In *spw1*(*osmads16*), all of the stamens are transformed into plos, whereas the *DL* mutation leads to pistil homeotically transformed into ectopic stamens (Nagasawa *et al.*, 2003; Xiao *et al.*, 2003). Therefore, *DL* acts antagonistically with *OsMADS16* in rice floral development (Nagasawa *et al.*, 2003; Yamaguchi *et al.*, 2004). In Ubi::

OsMADS16 transgenic lines, the pistil was replaced by stamen-like organs, and the stamen number was increased (Lee *et al.*, 2003). We therefore deduced that ectopic expression of *DL* might result in the transformation of stamens to pistils. In our *dfo1* mutants, the paleas had five vascular bundles and the lemma had an awn, whereas the stamens were partially or completely transformed into plos and some spikelets also had more than one pistil (Fig. 3). These results suggest that mutation of *DFO1* results in ectopic *DL* expression that probably affects the identities of the palea and the development of the awn, as well as stamens, in *dfo1* mutants.

Functional divergence between DFO1 and EMF1

DFO1 is orthologous to the *Arabidopsis* gene *EMF1*, which encodes a novel transcriptional regulator. In *Arabidopsis*, *EMF1* was expressed in all vegetative organs and flowers of all stages (Aubert *et al.*, 2001). Mutation of *EMF1* resulted in increased expression of *AG* and *CRABS CLAW* (Moon *et al.*, 2003). In rice, *DFO1* is also expressed in all vegetative organs and all four floral whorls (Fig. 7), and *dfo1* mutants have phenotypes partially similar to the *emf1* mutants in their floral organs (Chen *et al.*, 1997). In addition, the orthologs of *AG* and *CRABS CLAW* genes (*OsMADS3*, *OsMADS58* and *DL*) were also ectopically expressed in *dfo1-1* spikelets. These results indicate that *DFO1* negatively regulates C-class genes in both rice and *Arabidopsis*. Therefore, the functional similarity of both genes in the regulation of floral development suggests a conserved EMF1/DFO1-mediated pathway in both dicots and monocots.

In spite of these similarities, the function of *DFO1* in floral development and the flowering pathway may partially diverge from that of *EMF1*. First, the *Arabidopsis* A-class (*AP1*) and B-class (*AP3*, *PI*) genes were expressed ectopically in *emf1* mutants, whereas expression of the orthologs of these genes decreased in the *dfo1-1* mutant (Moon *et al.*, 2003) (Fig. 8). Second, *Arabidopsis emf1* mutants started to flower immediately after germination, whereas rice *dfo1* mutants grew for *c.* 3 months before flowering. Further studies have suggested that the early flowering phenotype in *emf1* mutants is probably a result of the ectopic expression of A/B/C/D-class genes or interaction with EIP proteins in *Arabidopsis* (Moon *et al.*, 2003; Sánchez *et al.*, 2009; Park *et al.*, 2011). However, in rice *dfo1* mutants, only the C-class genes were ectopically expressed, and they cannot form a flower. Therefore, we assume that the functional divergence of *EMF1* in the flowering time pathway and the mechanisms of regulation of floral organ identity genes are not strictly conserved between rice and *Arabidopsis*.

Role of *DFO1* in rice floral development

In *Arabidopsis*, EMF1 interacts with the EMF2-PRC2 complex (EMF2, MSI1, CLF and FIE) via MSI1, and also interacts with RINGs (homologs of *Drosophila* PRC1 complex), and probably plays a PRC1-like role or acts as a member of PRC2 in the repression of expression of its targets (Calonje *et al.*, 2008; Bratzel *et al.*, 2010; Kim *et al.*, 2012). However, the putative components of PRC1 and PRC2 in rice have not been identified, although it has been shown that OsiEZ1 can interact with OsFIE1 and OsFIE2, and OsCLF can interact with OsFIE1 in yeast (Zhang *et al.*, 2012). In our studies, we found that OsMSI1 also interacts with OsFIE1 in yeast (Fig. S6b). Thus, the equivalent of the PRC2 complex probably exists in rice.

In this study, our results verified that DFO1 could interact with OsMSI1 and OsiEZ1 (homologs of *Arabidopsis* PRC2). However, no interactions were found in yeast two-hybrid assay between DFO1 and OsRING or OsLHP1 proteins (homologs of *Arabidopsis* PRC1) (Fig. S6a). We therefore deduced that DFO1 might be a member of the putative rice PRC2 complex acting via the PcG mechanism. ChIP assays showed that the modification of H3K27me3 on *OsMADS58* and other C-class genes was changed in *dfo1-1* (Figs 10d–g, S7). Thus, DFO1 appeared to mediate the expression of C-class genes through epigenetic regulation. It will be intriguing to investigate whether DFO1 can interact directly with its targets, to obtain evidence of DFO1 function cooperating with PRC2 and H3K27me3 deposition, and to see whether DFO1 functions as a member of the PRC1-like complex *in vivo*. Future research will focus on the identification of the PRC1-like and PRC2-like components and the isolation of DFO1-containing complexes in rice to further reveal the role of DFO1 in the PcG-mediated gene silencing mechanism.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Spikelets of wild-type (WT) and *dfo1* mutants.

Fig. S2 Phenotypes of wild-type (WT) and *dfo1-1* plants.

Fig. S3 Alignment of DEFORMED FLORAL ORGAN1 (DFO1) with amino acid sequences of putative EMBRYONIC FLOWER1 (EMF1) proteins from other species.

Fig. S4 Alignment of DEFORMED FLORAL ORGAN1 (DFO1) and EMBRYONIC FLOWER1 (EMF1) amino acid sequences.

Fig. S5 Subcellular localization of DEFORMED FLORAL ORGAN1 (DFO1) fragments in rice protoplasts.

Fig. S6 Interaction between DEFORMED FLORAL ORGAN1 (DFO1) (Ct) and the polycomb repressive complex 1 (PRC1) component, and the interaction between OsMSI1 and OsFIE1 in yeast.

Fig. S7 Trimethylation of lysine 27 of histone H3 (H3K27me3) on *OsMADS3* and *DL* chromatin.

Table S1 Genetic analysis of wild-type and *dfo1-1*

Table S2 Primers used in mapping

Table S3 Primers used for vector construction in this study

Table S4 Primers used for real-time PCR analysis

Table S5 Primers used for chromatin immunoprecipitation (ChIP)-real-time PCR analysis

Table S6 Internode diameter of wild-type and *dfo1-1*

Table S7 Fourteen candidate open reading frames (ORFs) in the 60-kb genomic fragment

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