Running head: *MFS1* regulates spikelet development in rice

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MULTI-FLORET SPIKELET1, which encodes an AP2/ERF protein, determines spikelet meristem fate and sterile lemma identity in rice

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ABSTRACT

The spikelet is a unique inflorescence structure of grass. The molecular mechanism that controls the development of spikelet remains unclear. In this study, we identified a rice spikelet mutant, *multi-floret spikelet1* (*mfs1*), which showed delayed transformation of spikelet meristems to floral meristems, this resulted in an extra hull-like organ and an elongated rachilla. In addition, the sterile lemma was homeotically converted to the rudimentary glume and the body of the palea was degenerated in *mfs1*. These results suggest that the *MFS1* gene plays an important role in the regulation of spikelet meristem determinacy and floral organ identity. *MFS1* belongs to an unknown function clade in the AP2/ERF family. The MFS1-GFP fusion protein is localised in the nucleus. *MFS1* mRNA is expressed in various tissues, especially in the spikelet and floral meristems. Furthermore, our findings suggest that *MFS1* positively regulates the expression of *G1* and the *IDS1*-like genes *SNB* and *OsIDS1*. 
INTRODUCTION

In the reproductive phase of angiosperms, the shoot meristem is transformed into an inflorescence meristem, which then produces a floral meristem from which floral organs begin to develop, according to the mechanism known as the ABCDE model (Coen and Meyerowitz, 1991; Coen and Nugent, 1994; Dreni et al., 2007; Ohmori et al., 2009). An inflorescence can be classified as determinate or indeterminate based on whether its apical meristem is transformed into a terminal floral meristem. In an indeterminate inflorescence, the lateral meristem is permanently differentiated from the apical meristem, which is not converted into the terminal floral meristem, as occurs during the development of the inflorescences of Arabidopsis thaliana and Antirrhinum majus. In contrast, in a determinate inflorescence, the apical meristem is transformed into the terminal floral meristem after the production of a fixed number of lateral meristems, as occurs during the development of the inflorescences of Nicotiana tabacum and Solanum lycopersicum (Bradley et al., 1997; Ratcliffe et al., 1999; Sussex and Kerk, 2001; Chuck et al., 2008).

In general, inflorescences in grasses consist of branches and spikelets (Itoh et al., 2005; Coen and Nugent, 1994; Kaoru et al., 2010)). In these organisms, the branch meristem is determinate. It produces several lateral spikelet meristems, followed by the final production of a terminal spikelet meristem. The spikelet, the specific unit of the grass inflorescence, comprises a pair of bracts and 1–40 florets; it shows determinacy or indeterminacy depending on the species (Clifford, 1987; Malcomber et al., 2006). In species with a determinate spikelet, such as rice, after the production of fixed lateral floral meristems, the spikelet meristems are converted into terminal floral meristems, resulting in termination of the spikelet meristem fate. In contrast, in species with an indeterminate spikelet, such as wheat, the spikelet meristem fate is maintained continuously and produced a variable number of lateral floral meristems.

In Arabidopsis, the gene TERMINALFLOWER 1 (TFL1) was shown to maintain indeterminacy in the fate of inflorescence. In the tfl1 mutant, the inflorescence meristems were converted into floral meristems earlier than in the wild type, but the
ectopic expression of *TFL1* resulted in the transformation of floral meristems at a later stage of development to secondary inflorescence meristems (Bradley et al., 1997; Mimida et al., 2001; Ratcliffe et al., 1999). In rice, over-expression of either of the *TFL1*-like genes, *RICE CENTRORADIALIS1/2 (RCN1/2)*, delayed the transition of branch meristems to spikelet meristems, and finally resulted in the production of a greater number of branches and spikelets than in the wild type (Nakagawa et al., 2002; Rao et al., 2008).

To date, no gene that acts to maintain the indeterminacy of the spikelet meristem has been reported. However, two classes of genes have been shown to be involved in termination of the indeterminacy of spikelet meristems. One of these is the group of terminal floral meristem identity genes. A grass-specific *LEAFY HULL STERILE1 (LHS1)* clade in *SEPALLATA (SEP)* subfamily belongs to this class. *LHS1*-like genes were found to be expressed only in the terminal floral meristem in species with spikelet determinacy, which suggested that they exclusively determine the production of the terminal floral meristem, by which the spikelet meristem acquires determinacy (Cacharroón et al., 1999; Malcomber and Kellogg, 2004; Zahn et al., 2005). The other class comprises the *INDETERMINATE SPIKELET1 (IDS1)*-like genes, which belong to the *AP2/EREBP (AP2/ERF)* family. Unlike *LHS1*-like genes, this class of genes regulates the correct timing of the transition of spikelet meristem to floral meristem, but does not specify the identity of the terminal floral meristem. In maize, loss of *IDS1* function produce extra florets (Chuck et al., 1998). In addition, mutation of *SISTER OF IDS1 (SID1)*, a parologue of *IDS1* in maize, resulted in no defects in terms of spikelet development. However, the *ids1+sid1* double mutant failed to generate floral organs, but instead developed more bract-like structures than are found in wild-type plants (Chuck et al., 2008). The rice genome contains two *IDS1*-like genes, *SUPERNUMBERARY BRACT (SNB)* and *OsIDS1*. Loss of activity of *SNB* or *OsIDS1* produced extra rudimentary glumes, and *snb+osids1* double mutant developed more rudimentary glumes than either of its parental mutants (Lee et al., 2006; Lee and An, 2012). These results revealed that the mutated *IDS1*-like genes prolonged the activity of the spikelet meristem.
In most members of Oryzeae, the spikelet is distinct from those of other grasses, in that it comprises a pair of rudimentary glumes, a pair of sterile lemmas (empty glumes), and one floret (Schmidt and Ambrose, 1998; Ambrose et al., 2000; Kellogg, 2009; Hong et al., 2010). The rudimentary glumes are generally regarded as severely reduced bract organs, but the origin of sterile lemmas has been widely debated. Recent studies suggested that the sterile lemmas are the vestigial lemmas of two lateral florets. The gene *LONG STERILE LEMA* (*G1*)/*ELONGATED EMPTY GLUME1* (*ELE1*) is a member of a plant-specific gene family. In the *g1ele1* mutant, sterile lemmas were found to be homeotically transformed into lemmas (Yoshida et al., 2009; Hong et al., 2010). The *OsMADS34* and *EXTRA GLUME1* (*EG1*) genes were also shown to determine the identities of sterile lemmas. In the *osmads34* and *eg1* mutants, the sterile lemmas were enlarged and acquired the identities of lemmas (Li et al. 2009; Gao et al. 2010; Kaoru et al. 2010). Additionally, the *SEP*-like gene *LHS1/OsMADS1*, which specifies the identities of both the lemma and the palea, was not expressed in sterile lemmas, and ectopic expression in sterile lemmas resulted in the transformation of sterile lemmas to lemmas (Jeon et al. 2000; Li et al. 2009; Tanaka et al. 2012). These findings suggest that the sterile lemma may be homologous to the lemma. Nevertheless, some researchers still considered that the sterile lemmas are instead vestigial bract-like structures similar to the rudimentary glumes (Schmidt and Ambrose, 1998; Kellogg, 2009; Hong et al., 2010).

In the present study, we isolated the rice *MULTI-FLORET SPIKELET1* (*MFS1*) gene, which belongs to a clade of unknown function in the *AP2/ERF* gene family. The mutation of *MFS1* was shown to delay the transformation of the spikelet meristem to the floral meristem and to result in degeneration of the sterile lemma and palea. These results suggest that *MFS1* plays an important role in the regulation of spikelet determinacy and organs identity. Our findings also reveal that *MFS1* positively regulates the expression of *G1* and the *IDS1*-like genes *SNB* and *OsIDS1*.

**RESULTS**

We identified two recessive mutants related to the development of rice spikelet,
$mfs1-1$ and $mfs1-2$ (Fig 1, Supplemental Fig 1). An allelism test revealed that the two mutants were allelic. Given that $mfs1-1$ showed more severe defects than $mfs1-2$, the rest of this manuscript focuses primarily on the $mfs1-1$ mutant.

**$mfs1-1$ Shows Pleiotropic Defects in Spikelet Development**

Generally, a wild-type rice spikelet consists of one pair of rudimentary glumes, one pair of sterile lemmas and one terminal fertile floret. The floret comprises four whorls floral organs: one lemma and one palea in whorl 1, two lodicules in whorl 2, six stamens in whorl 3, and one pistil with two stigmas in whorl 4 (Fig. 1, A and B).

In wild-type spikelets, the sterile lemma was shown to be larger than the rudimentary glume (Fig. 1A). Most of the epidermis of the sterile lemma was smooth, and consisted of regularly arranged flat cells and rare cells with trichomes on the abaxial side (Fig. 1P). The epidermal cells of rudimentary glumes were arranged irregularly and bore lots of protrusions and trichomes (Fig. 1P). In contrast, the sterile lemma was found to be reduced to various degrees, even resembling the rudimentary glume in size in the $mfs1-1$ mutant (Fig. 1, F, I, L, and Supplemental Fig 1). The abundant protrusions and trichomes were borne on the middle and lower epidermis of degenerated sterile lemmas, which were highly similar to those of the rudimentary glume (Fig. 1, Q–T). Meanwhile, the regular and smooth cells, like those of the sterile lemma of wild type, were still remained on the top region of the degenerated sterile lemmas (Fig. 1, Q–T). These results indicated that the degenerated sterile lemma in the $mfs1-1$ mutant had the identities of both sterile lemmas and rudimentary glumes.

It was found that 65% of $mfs1-1$ spikelets developed an extra hull (lemma/palea)-like organ (Fig. 1, I, K and Supplemental Fig 1A). The wild-type lemma had four cell layers: silicified cells, fibrous sclerenchyma, spongy parenchymatous cells and nonsilicified cells, and developed five vascular bundles. Compared with the lemma, the palea had three vascular bundles and consisted of two parts: the body of the palea (bop) and two marginal regions of the palea (mrp). The cellular structure of the bop was very similar to that of the lemma, but the mrp displayed a distinctive smooth epidermis, which lacked the epicuticular silicified thickening found in the lemma and bop (Fig. 1, B–E). In the $mfs1-1$ mutant, the extra
hull-like organ showed a similar histological structure and had five vascular bundles, resembling the wild-type lemma (Fig. 1, J and N). We detected the mRNA levels of the lemma identity gene DROOPING LEAF (DL), the lemma and palea identity genes OsMADS1, OsMADS14 and OsMADS15, and the mrp identity gene OsMADS6 in mfs1-1 extra hull-like organs. Abundant levels of OsMADS1, OsMADS14, OsMADS15, and DL transcripts were detected, but no OsMADS6 expression was found (Fig. 1U). These results revealed that the extra hull-like organ had the identity of the lemma.

In mfs1-1 spikelets with extra lemma-like organs, 38% had no normal palea. Two palea-like organs were observed in the position normally occupied by the palea (Fig. 1, K, L and Supplemental Fig 1A). Interestingly, each palea-like organ consisted of two mrps and a smaller bop with two vascular bundles (Fig. 1, L–O). The mrp and bop each had a texture similar to that of the wild-type palea (Fig. 1, M and N). OsMADS1, OsMADS14, and OsMADS15 were expressed normally (Fig. 1U), whereas DL was not expressed in the palea-like organs, similar to the case for the wild-type palea (Fig. 1U). OsMADS6 expression was more intense in the mfs1-1 palea-like organs than in wild-type paleae (Fig. 1U). These findings suggested that the palea-like organs were degenerated paleae, and the increased OsMADS6 expression in the palea-like organs was probably caused by the relative abundance of mrp tissues.

In 21% of the mfs1-1 spikelets, the palea was degenerated to various degrees (Supplemental Fig 1A). Most of the degenerated palea contained the normal mrp and reduced bop (Supplemental Fig 1D and E). In a few occasions, the degenerated palea retained only mrp-like structures that contained a nonsilicified upper epidermis without trichomes and protrusions (Fig. 1, F, G and H). These results suggested that the development of mfs1-1 bop was severely affected.

Simultaneously, we also investigated the defects of the inner three whorls in the mfs1-1 mutant. In the florets (41%) with normal paleae, the identities and numbers of organs of the inner three whorls were not changed (Supplemental Table 2). In the florets (59%) with degenerated paleae, the numbers of organs of the inner three whorls were varied, but they retained their identities (Supplemental Table 2 and
Additionally, 81% of mfs1-1 spikelets possessed elongated rachillae (Fig. 1, F, I, Q, S and Supplemental Fig1A).

**mfs1-1 Exhibited Abnormal Early Spikelet Development**

We examined young spikelets of the wild type and mutant at different developmental stages by scanning electron microscropy (SEM). During the spikelet 4 stage (Sp4), lemma and palea primordia of the wild-type flower started to develop, and the lemma had a bumped top and was larger than the palea (Fig. 2A). In the mfs1-1 mutant, some spikelets developed extra lemma-like organ primordia, and their paleae were either normal (Fig. 2I) or degenerated (Fig. 2M). At the same time, the floral meristem was enlarged in parts of the spikelets with degenerated paleae (Fig. 2M). Other spikelets had no extra lemma-like organ, whereas their palea primordia were reduced in size (Fig. 2E). During Sp5 and Sp6, the wild-type flower formed six spherical stamen primordium, the development of one stamen primordia on the lemma side was delayed, whereas the others developed synchronously (Fig. 2B). No significant differences were observed in those florets with an extra lemma-like organ and normal palea (Fig. 2J). However, in some florets with extra lemma-like organs and abnormal paleae, stamen development was not synchronous and the number of stamens varied (Fig. 2, N and O, Supplemental Fig 1 F and G, Supplemental Table 2). In the florets with degenerated paleae, we found no obvious defects except in terms of the number of stamens (Fig. 2F, Supplemental Table 2). At the Sp7 and Sp8 stages (formation of pistil primordia), the lemma and palea progressed to a further stage of development. In the mfs1-1 mutant, apparent extra lemma-like organs and degenerated paleae were observed (Fig. 2, H, L and P).

We also examined the sterile lemma at different developmental stages. At the Sp4–Sp6 stages, the sterile lemma was larger than the rudimentary glume in the wild type (Fig 3A and B). In the mfs1-1 mutant, the size of the sterile lemma was similar to that of the rudimentary glume (Fig 3E and F). At the Sp7–Sp8 stages, the sterile lemma differentiated drastically and was much larger than the rudimentary glume in the wild type (Fig 3C and D). However, the mfs1-1 sterile lemma was smaller than that of the wild type, resembling the rudimentary glume at these stages (Sp7 and Sp8).
Meanwhile, the epidermal cells started to elongate in sterile lemmas and still maintained their size in the rudimentary glume in the wild type (Fig 3D), whereas their sizes were maintained in both the rudimentary glume and the sterile lemma of the mfs1-1 mutant (Fig 3H). These results suggested that the identity of the mfs1-1 sterile lemma was affected, and the sterile lemma in the mfs1-1 mutant displayed a development pattern similar to that of the rudimentary glume.

**Expression Patterns of Floral Organ Identity Genes during Early Stages of Flower Development**

The expression patterns of DL, OsMADS1, and OsMADS6, which are known to be involved in the regulation of the lemma and palea identities, were investigated during the early stages of flower development.

In wild-type flowers, DL was first expressed in the lemma primordia at the stages Sp4–Sp6 (Fig. 4, A and B), and then also in pistil primordia after SP7 (Fig. 4C), whereas DL transcripts were still retained in the lemma at Sp8 (Fig. 4D). In mfs1-1 flowers, the DL signals were pronounced in extra lemma-like organ, besides the lemma and pistil (Fig. 4, E–H). These findings proved that the spikelets did indeed developed extra lemmas at the early stage of flower development.

stages Sp4–Sp8, OsMADS1 was expressed in the lemmas and paleae of wild-type florets (Fig. 5, A–D). In the mfs1-1 mutant, OsMADS1 signals were observed in the extra lemma-like organ, lemma and palea (Fig. 5, E–H).stages Sp4–Sp7, OsMADS6 expression exhibited no significant differences between mfs1-1 and wild-type flowers, and was detected in the floral meristem and primordia of the mrp, lodicule, and pistil (Fig. 5, I–K and M–O). At Sp8, in the transverse section of wild-type florets, OsMADS6 transcripts were found in the mrp, lodicule, and pistil (Fig. 5L). In the mfs1-1 mutant, OsMADS6 signals were detected in the two mrps of each palea-like organ, lodicule, and pistil (Fig. 5P). These results further suggested that the palea-like organs were derived from paleae in the mfs1-1 mutant.

**Molecular Cloning and Identification of MFS1**

The MFS1 gene was previously mapped to a region of about 350 kb on chromosome 5 (Ren et al., 2012). Here, the location of MFS1 was narrowed down to
within a physical distance of 67 kb between the indel markers IND17 and IND24 (Fig. 6A), in which there are 16 annotated genes (http://www.gramene.org/). Sequencing analysis identified a single nucleotide substitution from C to T within a predicted AP2/ERF transcription factor (LOC_Os05g041760) in different positions of the two mfs1 alleles, causing amino acid mutations of Ala66 to Val66 in the mfs1-1 mutant and Thr51 to Ile51 in the mfs1-2 mutant (Fig. 6A). To test whether these mutations were causally linked to the mutant phenotype, the Os05g041760 wild-type genomic fragment that contained the coding sequence, 2,925 bp of sequence upstream of the start codon, and 938 bp of sequence downstream of the stop codon was transformed into mfs1-1. As a result of this, the mutant phenotypes were completely rescued in transgenic plants (Supplemental Fig 2, A–C). We further performed RNA interference to silence MFS1 in the japonica cultivar ZH11. In the transgenic plants, the level of MFS1 transcript was greatly reduced (Fig. 6F) and pleiotropic spikelet defects similar to those of mfs1-1 were observed (Fig. 6, B–E). Taken together, these results confirmed that the Os05g041760 is the MFS1 gene.

**MFS1 Encodes an ERF Domain Protein**

The AP2/ERF gene family is plant-specific and includes four subfamilies: AP2, RAV, DREB and ERF (Sharoni et al. 2011). Phylogenetic analysis showed that MFS1 and its orthologs from moss, gymnosperms, dicots, and grasses constitute an MFS1-like clade, whereas the well-known ERF domain protein FZP and BD1, and their orthologs constitute another clade in the ERF subfamily (Fig. 7). These results suggested that MFS1-like and FZP/BD1-like genes diverged before the emergence of gymnosperms and the MFS1-like genes differ from the well-known AP2/ERF genes. In addition, phylogenetic analysis also showed that the other known AP2 domain genes (SNB, OsIDS1 and SHAT1) have a distant evolutionary relationship with the MFS1-like and FZP/BD1-like genes.

Sequence analysis showed that all MFS1-like proteins contain a highly conserved ERF domain, located close to their N-terminus. Meanwhile, a conserved C-terminus domain was identified in MFS1-like proteins from grasses and dicots, which share the DLNEPP^{185-190} motif. A unique sites (V^{37}) and a motif (SPWH^{132-135}) were also
identified in MFS1-like proteins from grasses (Supplemental Fig 3). In addition, the 
MFS1 gene shared low sequence similarity with the known AP2/ERF genes outside 
the AP2/ERF domain (Supplemental Fig 3).

Vectors that contained the MFS1ORF-GFP fusion protein, the SL1ORF-GFP 
fusion protein and the single GFP protein were transiently expressed in rice 
protoplasts. The SL1ORF-GFP protein acted as a positive nuclear gene control (Xiao 
et al., 2009). Green fluorescence was detected in the nuclei of rice protoplasts for both 
MFS1ORF-GFP and SL1ORF-GFP fusion proteins (Fig. 6, J–L and Supplemental Fig 
2, D–F). In cells that expressed GFP alone, green inflorescence was observed 
uniformly throughout in the cell, apart from in the vacuole (Fig. 6, G–I). These results 
suggest that MFS1 encodes a nuclear protein, that may act as a transcription factor.

Expression Patterns of MFS1

Quantitative RT-PCR (qPCR) analysis showed that MFS1 was universally 
expressed in various tissues including roots, stems, leaves and panicles, with higher 
levels in young panicles (≤2 cm) than in the other tissues examined (Fig. 8A). 
Furthermore, the MFS1 expression pattern was investigated by in situ hybridization. 
First, MFS1 was highly expressed in the meristems of branches and spikelets (Fig. 8, 
B, C and D). Next, strong signals were observed at the sites of initiation of the sterile 
lemma primordium (Fig. 8E). When the lemma and palea primordia formed, abundant 
MFS1 transcripts were detected in the lemma, palea, and floral meristem (Fig. 8, D, F 
and G). Subsequently, the expression of MFS1 was primarily restricted to the lemma, 
palea, lodicule, and stamen (Fig. 8, G and H). After the formation of pistil, MFS1 
signals disappeared from the lemma and palea, but were retained in the lodicule, 
stamen, and pistil (Fig. 8, I and J).

MFS1 Affects the Expression of Genes Related to Spikelet Development

Given that mfs1-1 mutant exhibited spikelet defects, we examined the expression 
levels of the IDS1-like genes SNB and OsIDS1, which are closely associated with the 
transition and determinacy of spikelet meristem in rice (Lee et al., 2006, Lee and An, 
2012). SNB transcripts accumulated primarily in young panicles less than 2 cm long,
and their levels were lower in the *mfs1-1* mutant than in the wild type (Fig. 9A). Then, levels of *SNB* transcripts were dramatically decreased in panicles longer than 2 cm, and no difference in the levels of *SNB* expression was found between wild-type and *mfs1-1* panicles with a length 2–5 cm (Fig. 9A). *OsIDS1* transcripts were first detected in young panicles less than 0.5 cm, and then were more abundant in wild-type panicles between 0.5–5 cm length (Fig. 9A). Compared with that in the wild type, *OsIDS1* expression showed no obvious change in panicles with a length less than 0.5 cm, whereas it dramatically decreased in *mfs1-1* panicles 0.5 to 5 cm long (Fig. 9A). These results imply that *MFS1* positively regulated the expression of the *IDS1*-like genes *SNB* and *OsIDS1*.

We used the qPCR to examine the expression of the *G1* gene which has been shown to be involve in the specification of sterile lemma identity (Yoshida et al., 2009; Hong et al., 2010). In the wild type, a high level of *G1* expression was detected in panicles shorter than 2 cm, but the mRNA levels were significantly reduced in those that were 2 to 5 cm (Fig. 9A). In the *mfs1-1* mutant, *G1* showed lower expression levels in young panicles shorter than 5 cm (Fig. 9A). *In situ* hybridization indicated that in the wild type, the *G1* signals were strongly detected in sterile lemmas during the stage of the sterile lemma primordia differentiation and formation, and subsequently decreased markedly when sterile lemmas started to elongate (Fig. 9, B–F). *G1* expression was faint in the sterile lemma primordia of *mfs1-1* spikelet during the stages analysed (Fig. 9, G–K), which was consistent with the results of qPCR analysis. These findings suggest that *MFS1* positively regulates *G1* expression.

**DISCUSSION**

In this study, we characterized a novel AP2/ERF domain gene *MFS1*, which is involved in the regulation of spikelet meristem determinacy and floral organ identity in rice. *MFS1* promotes the expression of the *SNB*, *OsIDS1*, and *G1* genes involved in the development of spikelets.

**MFS1 Affects Spikelet Meristem Determinacy**

Most spikelets in *mfs1-1* mutant plants each developed an extra lemma. About 27%
of these spikelets produced normal florets after the extra lemmas arose, which suggested that these spikelets were composed of a terminal floret and a degenerated lateral floret that only contained the lemma. In the other spikelets with abnormal florets, two palea-like organs were observed, which corresponded to the extra lemma and the original lemma. Together with the development of a floral meristem that reached a larger than usual size at an earlier stage (Fig. 2M), these results imply that these spikelets tended to produce two florets, and the spikelet meristem determinacy was disturbed in the mfs1-1 mutant. Similarly, in the tongari-boushi1 (tob1) mutant, some spikelets had an extra lemma/palea-like organ between the sterile lemma and the original lemma (Tanaka et al., 2012). In the snb mutant, some spikelets developed supernumerary rudimentary glumes, extra lemma- or palea-like structures, or lateral florets before the terminal floret emerged (Lee et al., 2006). The snb+osids1 double mutant even produced more bract-like organs, including rudimentary glumes, lemmas or paleae, than single mutants (Lee and An, 2012). These results suggest that SNB, OsIDS1, TOB1 and MFS1 regulate the fate of the spikelet meristem by ensuring the correct timing of the transition of the spikelet meristem to terminal floral meristem. In contrast, loss of the spikelet meristem determinacy occurred before the formation of rudimentary glume in snb and osids1 mutants, but after the emergence of sterile lemmas in tob1 and mfs1-1 mutants. This suggested that MFS1 and TOB1 function later than SNB and OsIDS1. Additionally, decreases in the expression of SNB and OsIDS1 were found in mfs1-1 mutant young panicles, which suggested that MFS1 positively regulated the expression of SNB and OsIDS1.

**MFS1 Specifies Sterile Lemma Identity**

In the mfs1-1 mutant, the degenerated sterile lemma exhibited the identities of both the sterile lemma and the rudimentary glume. In the snb mutant, no sterile lemmas were found at sites where extra rudimentary glumes were present (Lee et al., 2006), which suggested the homeotic transformation of the sterile lemma to the rudimentary glume. In the osids1 mutant, one of the sterile lemmas was shown to be occasionally replaced by a rudimentary glume (Lee and An, 2012). SNB and OsIDS1 were also previously shown to encode an AP2/ERF domain protein (Lee et al., 2006, Lee and
These results suggest that \textit{MFS1}, \textit{SNB} and \textit{OsIDS1} confer important functions in the development of the sterile lemma. It was reported that \textit{G1/ELE}, \textit{OsMADS34}, and \textit{EG1} determined the identity of the sterile lemma. In these mutants, the sterile lemma was homeotically transformed into the lemma (Yoshida et al., 2009; Hong et al., 2010; Li et al. 2009; Gao et al. 2010; Kaoru et al. 2010). These results suggest that \textit{G1/ELE}, \textit{OsMADS34}, and \textit{EG1} prevent the transformation of the sterile lemma to the lemma, whereas \textit{MFS1}, \textit{SNB}, and \textit{OsIDS1} prevent the degeneration of the sterile lemma to the rudimentary glume.

There have been two prevailing hypotheses on the origin and evolution of the sterile lemma (Takeoka et al., 1993). One states that a putative ancestor of \textit{Oryza} had a spikelet that contained a terminal floret and two lateral florets, which subsequently degenerated during evolution, leaving only the lemma (Arber, 1934; Kellogg, 2009). The sterile lemma would thus seem to be derived from morphological modification of the remnants of this lemma (Yoshida et al. 2009; Kobayashi et al., 2010). The other hypothesis suggests that the spikelet of \textit{Oryza} species has only one floret, and the sterile lemma and rudimentary glume are universally regarded as severely reduced bract structures (Schmidt and Ambrose, 1998; Terrell et al., 2001; Hong et al., 2010). In the \textit{g1/ele1}, \textit{osmads34}, and \textit{eg1} mutants, the sterile lemmas were enlarged and transformed into lemmas, which supports the first hypothesis. In the \textit{mfs1-1} mutant, the sterile lemma was degenerated and acquired the identity of the rudimentary glume, which supports the second hypothesis. In fact, in most grass species, the spikelet lacks sterile lemma-like organs, and only contains one or more florets and bract-like glume organs, which are considered to be equivalent to the rudimentary glumes of \textit{Oryza} species (Takeoka et al., 1993; Yoshida et al., 2009; Hong et al., 2010). The bract-like glume organ resembles the lemma in size and structure in some grass species, such as maize and wheat (Kellogg, 2001; Yoshida et al., 2009), whereas it is severely reduced in \textit{Oryza} species (Bommert et al., 2005; Li et al., 2009). Therefore, the lemma, sterile lemma and rudimentary glume may be homologous structures.

\textit{MFS1} Regulates the Palea Development
In grass flowers, the palea was thought to have a different identity and origin from the lemma. In general, the palea is considered homologous to the prophyll (the first leaf produced by the axillary meristem) that is formed on a floret axis, whereas the lemma corresponds to the bract (the leaf subtending the axillary meristem) that is formed on a spikelet axis (Kellogg, 2001; Ohmori et al., 2009). Recently, some evidence has indicated that the rice palea is an organ produced by congenital fusion of the bop and the mrp, which potentially have distinct origins (Francis, 1920; Cusick, 1966; Verbeke, 1992; Zanis, 2007). Specifically, first, the cellular structure of the bop was shown to be highly similar to that of the lemma, but distinct from that of the mrp (Prasad et al., 2005; Sang et al., 2012). Second, the rice B-class mutant superwoman1 (spw1/osmads16) and MADS2+MADS4 double RNAi plants showed transformation of the lodicules into organs that resembled the mrp but not the bop. Moreover, mutations of Arabidopsis B-class genes undergo homeotic transformation of petals (equivalent to lodicules) into sepals (Nagasawa et al., 2003; Yadav et al., 2007; Yao et al., 2008). These findings suggest that the mrp, but not bop, is homologous to the sepal. In the depressed palea1 (dp1) mutant, the palea was shown to be replaced by two mrp-like structures and the bop was lost (Luo et al., 2005; Jin et al., 2011). In the retarded palea1 (rep1) mutant, the development of the bop was delayed, whereas over-expression of REP1 caused over-differentiation of the mrp cells (Yuan et al., 2009). In the mfs1-1 mutant, the bop was degenerated in most florets and was even absent in a few cases. Additionally, recent studies revealed that CHIMERIC FLORAL ORGANS 1 (CFO1/OsMADS32) and MOSAIC FLORAL ORGANS1 (MFO1/OsMADS6) were expressed in the mrp, the mutations of which resulted in transformation of the mrp into lemma-like or bop structures (Ohmori et al., 2009; Li et al., 2010; Sang et al., 2012). These results indicated that two parts of the palea are controlled by different regulatory pathways. Whereas MFS1, DP1 and REP1 determine the bop identity, MFO1 and CFO1 are involved in regulation of the mrp identity. Consistent with these hypotheses, the phenotypes of the mfs1 palea suggest that the rice palea is an organ produced by the fusion of the mrp and bop, which have different origins.
CONCLUSION

In this study, we characterized the rice *MULTI-FLORET SPIKELET1* (*MFS1*) gene, which belongs to a clade of unknown function in the *AP2/ERF* gene family. The *mfs1* spikelets displayed extra lemmas, degenerated sterile lemmas and paleae. These results suggest that *MFS1* plays an important role in the regulation of spikelet determinacy and organs identity. Our data also reveal that *MFS1* positively regulates the expression of *G1* and the *IDS1*-like genes *SNB* and *OsIDS1*.

MATERIALS AND METHODS

Plant Materials

Two mutants of rice (*Oryza sativa* L), *mfs1-1* and *mfs1-2*, were identified from ethylmethane sulfonate (EMS)-treated Jinhui 10. The Jinhui 10 cultivar was used as a wild-type strain for phenotypic observation. All plants were cultivated in paddies in Chongqing and Hainan, China.

Map-based Cloning of *MFS1*

The *mfs1-1* mutant was crossed with Xinong1A (*Oryza sativa* L), and 1,418 F2 plants with the mutational phenotype were selected and used as a mapping population. Initial gene mapping was conducted using simple sequence repeat (SSR) markers from public available rice databases, including Gramene (http://www.gramene.org) and Rice Genomic Research Program (http://rgp.dna.affrc.go.jp/publicdata/caps/index.html). Then, fine mapping was performed using developed insertion and deletion (InDel) markers from comparisons of genomic sequences from Xinong1A and Jinhui 10 in our laboratory. The sequences of primers used in the mapping and candidate gene analysis are listed in Supplemental Table 1.

Microscopy Analysis

Panicles were collected at different developmental stages and fixed in 50% ethanol, 0.9 M glacial acetic acid and 3.7% formaldehyde overnight at 4°C, dehydrated with a graded ethanol series, infiltrated with xylene, and embedded in paraffin (Sigma). The
8-μm-thick sections were transferred onto poly-L-lysine-coated glass slides, deparaffinized in xylene and dehydrated through an ethanol series. The sections were stained sequentially with 1% safranine (Amresco) and 1% Fast Green (Amresco), then dehydrated through an ethanol series, infiltrated with xylene and finally mounted beneath a cover slip. Light microscopy was performed using a Nikon E600 microscope. For scanning electron microscopy, fresh samples were examined using a Hitachi S-3400 scanning electron microscope with a –20°C cool-stage. The stages of early spikelet development were the same as those defined previously (Ikeda et al. 2004).

**RNA Isolation and Quantitative RT-PCR Analysis**

RNA from root, stem, leaf, inflorescence and young flowers was isolated using the Watson RNeasy Plant Mini Kit (Watson). The first strand of cDNA was synthesized from 2 μg of total RNA using oligo(dT)18 primers in a 25 μl reaction volume using the SuperScript III Reverse Transcriptase Kit (Invitrogen). Half a microliter of the reverse-transcribed RNA was used as a PCR template with gene-specific primers (Supplemental Table 3). The qRT-PCR analysis was performed with an ABI Prism 7000 Sequence Detection System and the SYBR Supermix Kit (Bio-Rad). At least three replicates were performed and the mean values of the expression of each gene were used.

**In Situ Hybridization**

The 482-bp gene-specific *MFS1* probe was amplified with the primers *MFS1*-F and *MFS1*-R and labeled using the DIG RNA Labelling Kit (SP6/T7) (Roche). Probes for the known floral organ genes were prepared using the same method. Pretreatment of sections, hybridization and immunological detection were performed as described previously (Sang et al., 2012). The primer sequences are listed in Supplemental Table 1.

**Vector Construction**

For the complementation test, a 4,433-bp genomic fragment that contained the *MFS1* coding sequence, coupled with the 2925-bp upstream and 938-bp downstream sequences, was amplified using the primers *MFS1*com-F and *MFS1*com-R. The
resulting PCR products were digested using XbaI and EcoRI, and then inserted into the binary vector pCAMBIA1301. The recombinant plasmids were introduced into mfs1-I by the Agrobacterium-mediated transformation method as described previously (Sang et al., 2012). To make a construct for RNAi, we amplified a 267-bp fragment of MFS1 cDNA with the primers MFS1/Ri-F (SpeI, KpnI) and MFS1/Ri-R (SacI, BamHI), as shown in Supplemental Table 1. The resulting PCR products were first digested using SpeI and SacI, and then ligated into vector pTCK303 (Wang et al., 2004) to obtain the intermediate vector. The PCR products were then digested using KpnI and BamHI, and ligated into the intermediate vector. The recombinant plasmids were transformed into ZH11 plants by the Agrobacterium-mediated transformation method. The primer sequences are listed in Supplemental Table 1.

Subcellular Localization

The coding region of MFS1 without the stop codon was amplified using the primer pair MFS1OE-F/MFS1OE-F, which contain XbaI and BamHI site, respectively (Supplemental Table 1). The fragment was cloned into the expression cassette 35S-GFP (S65T)-NOS (pCAMBIA1301) with appropriate modifications, which generated the MFS1-GFP fusion vector. The GFP and MFS1-GFP plasmids were transformed into rice protoplasts as described previously (Li et al., 2009). After 8–16 h of incubation at 28°C, GFP fluorescence was observed with a Nikon E600 microscope.

Protein Sequence and Phylogenetic Analysis

Protein sequences were obtained by searching GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the MFS1 sequence as a query. A phylogenetic tree was constructed using MEGA 5.0 (Tamura et al., 2011). The tree was constructed using the maximum likelihood method based on the JTT matrix-based model with the lowest Bayesian Information Criterion scores (Jones et al., 1992; Tamura et al., 2011). Bootstrap support values for each node from 500 replicates are shown next to the branches (Felsenstein, 1985). The initial tree for the heuristic search was obtained automatically as follows. When the number of common sites was < 100 or less than one-quarter of the total number of sites, the maximum
parsimony method was used; otherwise Bio-neighbor-joining method with Markov Cluster distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6362). The tree was drawn to scale, with branch lengths measured in terms of the number of substitutions per site.

Supplemental Data
Supplemental Table 1. Primers used in the study.
Supplemental Table 2. Distribution of the number of foral organs in the wild type and mfs1-1
Supplemental Figure 1. Investigation of mfs1 spikelets.
Supplemental Figure 2. Complementation test and subcellular localization.
Supplemental Figure 3. Protein sequence alignment of the closely related AP2/ERF genes.

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Figure legends

Figure 1. Phenotypes of spikelets in the wild-type and mfs1-1. A and B, wild-type spikelet. C-1, epidermal surface of wild-type palea. C-2, epidermal surface of wild-type lemma. D and E, histological analysis of wild-type spikelet. F and G, mfs1-1 spikelet with a degenerated palea. H, epidermal surface of the degenerated palea in (G). I, mfs1-1 spikelet with an extra hull and a normal palea. J, histological analysis of mfs1-1 spikelet with an extra hull and a normal palea. K and L, mfs1-1 spikelet with an extra hull and degenerated paleae. M, epidermal surface of the
degenerated palea in (L). N and O, histological analysis of an *mfs1-1* spikelet with an extra hull and degenerated paleae. P, epidermal surface of the sterile lemma and rudimentary glume in the wild type. Q–T, epidermal surface of the degenerated sterile lemma and rudimentary glume in *mfs1-1*. U, relative expression levels of floral organ identity genes in the wild-type and *mfs1-1* floral organs. rg, rudimentary glume; sl, sterile lemma; le, lemma; hl, hull (lemma/palea)-like organ; pa, palea; lo, lodicule; st, stamen; pi, pistil; bop, body of palea; mrp, marginal region of palea; dsl, degenerated sterile lemma; dp, degenerated palea; er, elongated rachilla; pal, palea-like organ. Black arrows represent vascular bundles. Bars = 1000 μm in A, B, F, G, I, K and L, and 100 μm in C–E, H, J, M and N–T. Error bars indicate SD.

**Figure 2.** Spikelets at early developmental stages in the wild type and *mfs1-1*. A–D, wild type spikelet. (A) Sp4, (B) Sp5–6, (C) Sp7, (D) Sp8. E–P, *mfs1-1* spikelet. (E, I and M) Sp4, (F, J, and N) Sp5–6, (G, K, and O) Sp7. (H, L, and P) Sp8. fm, floral meristem; sl, sterile lemma; le, lemma; el, extra lemma-like organ; pa, palea; lo, lodicule; pi, pistil; dsl, degenerated sterile lemma; pal, palea-like organ. Asterisks indicate the stamens. Bars = 100 μm.

**Figure 3.** Sterile lemma development in wild-type and *mfs1-1* spikelet at early stages. A–D, development of the sterile lemma in the wild type. (A) Sp4, (B) Sp5–6, (C) Sp7, (D) Sp8. E–H, the sterile lemma development in *mfs1-1*. (E) Sp4, (F) Sp5–6, (G) Sp7, (H) Sp8. rg, rudimentary glume; sl, sterile lemma; le, lemma; hl, hull-like organ; pa, palea; pal, palea-like organ; dsl, degenerated sterile lemma. Bars = 100 μm.

**Figure 4.** Expression of *DL* gene in the wild-type and *mfs1-1* flowers. A–D, wild-type flowers. E–H, *mfs1-1* flowers. Rows 1–3 show longitudinal sections of flowers at stages Sp5 to Sp7, respectively. Row 4 shows transverse sections of flowers at the Sp8 stage. le, lemma; pa, palea; pi, pistil; pal, palea-like organ, vb, vascular bundle; el, extra lemma-like organ. Bars = 50 μm.

**Figure 5.** Expression of *OsMADS1* and *OsMADS6* in wild-type and *mfs1-1* flowers. A–D, *OsMADS1* expression in the wild-type flowers. E–H, *OsMADS1* expression in the *mfs1-1* flowers. I–L *OsMADS6* expression in the wild-type flowers. M–P, *OsMADS6* expression in the *mfs1-1* flowers. Rows 1–3 show longitudinal sections of
flowers at stages Sp5 to Sp7, respectively. Row 4 shows transverse sections of flowers at the Sp8 stage. fm, floral meristem; le, lemma; pa, palea; lo, lodicule; pi, pistil; pal, palea-like organ; mrp, marginal region of palea; el, extra lemma-like organ. Bars = 50 μm.

**Figure 6.** Isolation of the *MFS1* gene and subcellular localization of the MFS1 protein. A, map position of the *MFS1* locus. The relative positions of BAC clones are shown. Genomic structure of *MFS1*. The sites of the mutation in *mfs1* are shown. B, phenotype of ZH11 plants. C–F, RNAi analysis of *MFS1* and phenotypes of transgenic plants. C–E, phenotypes of RNAi transgenic plants; F, *MFS1* expression in RNAi transgenic plants. G–L, analysis of subcellular localization of MFS1 protein using rice protoplasts. G–I, GFP fusion protein. G, Digital Image Control (DIC) image; H, bright-field; I, merged GFP fusion protein. J–L, MFS1-GFP. J, DIC image; K, bright-field image; L, merged image of MFS1-GFP fusion protein. An arrow indicates the site of predicted genes in the IND17 to IND24 interval. le, lemma; hl, hull (lemma/palea)-like organ; pa, palea; sl, sterile lemma; er, elongated rachilla; dp, degenerated palea. Green arrows indicate the degenerated sterile lemma. Bars = 1000 μm in B–E, and 50 μm in G–L.

**Figure 7.** Phylogenetic tree of the MFS1 proteins. The phylogenetic tree was constructed using the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model. Gy/Mo, gymnosperms and mosses.

**Figure 8.** Expression pattern of *MFS1*. A, *MFS1* expression in different tissues as detected by qPCR. B–J, *In situ* hybridization in wild-type panicles and flowers, using an *MFS1* antisense probe. bm, branch meristem; sm, spikelet meristem; fm, floral meristem; rg, rudimentary glume; sl, sterile lemma; le, lemma; pa, palea; lo, lodicule; pi, pistil; R, root; S, stem; L, leaf. Asterisks indicate the stamens. Bars = 50 μm.

**Figure 9.** Expression of *SNB, OsIDS1* and *G1* in wild-type and *mfs1-1* flowers. A, qRT-PCR analysis of *SNB, OsIDS1* and *G1* in developing wild-type and *mfs1-1* panicles at different stages. B–F, *G1* expression in the wild-type flowers. G–K, *G1* expression in *mfs1-1* flowers. sm, spikelet meristem; sl, sterile lemma; le, lemma; pa, palea; lo, lodicule. Bars = 50 μm.