MOSAIC FLORAL ORGANS1, an AGL6-Like MADS Box Gene, Regulates Floral Organ Identity and Meristem Fate in Rice

Shinnosuke Ohmori, Mayumi Kimizu, Maiko Sugita, Akio Miyao, Hirohiko Hirochika, Eiji Uchida, Yasu Nagato, and Hitoshi Yoshida

a Rice Biotechnology Research Subteam (Hokuriku Region), National Agricultural Research Center, National Agriculture and Food Research Organization, Niigata 943-0193, Japan
b Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan
c Division of Genome and Biodiversity Research, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan

Floral organ identity and meristem determinacy in plants are controlled by combinations of activities mediated by MADS box genes. AGAMOUS-LIKE6 (AGL6)-like genes are MADS box genes expressed in floral tissues, but their biological functions are mostly unknown. Here, we describe an AGL6-like gene in rice (Oryza sativa), MOSAIC FLORAL ORGANS1 (MFO1/ MADS6), that regulates floral organ identity and floral meristem determinacy. In the flower of mfo1 mutants, the identities of palea and lodicule are disturbed, and mosaic organs were observed. Furthermore, the determinacy of the floral meristem was lost, and extra carpels or spikelets developed in mfo1 florets. The expression patterns of floral MADS box genes were disturbed in the mutant florets. Suppression of another rice AGL6-like gene, MADS17, caused no morphological abnormalities in the wild-type background, but it enhanced the phenotype in the mfo1 background, indicating that MADS17 has a minor but redundant function with that of MFO1. Whereas single mutants in either MFO1 or the SEPALATA-like gene LHS1 showed moderate phenotypes, the mfo1 lhs1 double mutant showed a severe phenotype, including the loss of spikelet meristem determinacy. We propose that rice AGL6-like genes help to control floral organ identity and the establishment and determinacy of the floral meristem redundantly with LHS1.

INTRODUCTION

Angiosperm flowers are quite diverse in their morphology. To establish such diverse morphologies, the specification of floral organ identity and control of floral meristem fate are critical. In eudicots, flowers generally consist of sepals (whorl 1), petals (whorl 2), stamens (whorl 3), and pistils (whorl 4) from the outer to inner whorls. Intensive molecular and genetic analyses in several eudicot species, notably Arabidopsis thaliana, snapdragon (Antirrhinum majus), and petunia (Petunia hybrida), established the so-called ABCDE model, in which combinations of the A/B/C/D/E functions specify the identity of each organ and control floral meristem determinacy (Coen and Meyerowitz, 1991; Theissen and Saedler, 2001). The spatially and temporally regulated expression patterns of the A/B/C/D/E genes and the complicated interaction patterns of their encoded proteins, which define organ identity and patterning, are the molecular basis for flower development. This robust genetic model is applicable to diverse plant species, not only eudicots but also monocot species, including some grass species, such as maize (Zea mays) and rice (Oryza sativa), at least in part (Ambrose et al., 2000; Nagasawa et al., 2003; Yamaguchi et al., 2006; Dreni et al., 2007).

The grasses, Poaceae, one of the largest monocot families, have floral architectures distinct from those of eudicots. The structural units of grass flowers are spikelets and florets. The spikelet comprises a series of bract-like organs, glumes, and florets. In rice, a single spikelet consists of two pairs of sterile glumes (rudimentary glumes and empty glumes) and one floret, and a single floret is comprised of one lemma, one palea, two lodicules (the equivalent of petals), six stamens, and one pistil (Bommert et al., 2005; Itoh et al., 2005). In contrast with Arabidopsis, in which the sepal is the whorl-1 organ, the identities of the outer floret organs of grasses (lemma and palea) are controversial, and the definition of the whorl-1 organ is not established in rice. In this article, we refer to the lodicule, stamen, and pistil/ovule whorls as whorls 2, 3, and 4, respectively, in accordance with previous studies (Nagasawa et al., 2003; Yamaguchi et al., 2006). In Arabidopsis, a heterodimer comprising two B-class MADS box proteins, APETALA3 (AP3) and PISTILLATA (PI), specifies the identities of the whorl-2 and -3 organs, the petal and stamen. In rice, either a loss-of-function mutation in Os SUPERWOMAN1 (SPW1/MADS16), the sole rice ortholog of At AP3, or the concomitant suppression of Os MADS2 and MADS4, two partially redundant rice orthologs of At PI, causes homeotic transformation of the lodicule (the whorl-2 organ in rice) and stamen into a palea-like organ and a carpel, respectively, indicating that the B-class function is conserved between Arabidopsis and rice (Nagasawa et al., 2003; Prasad and Vijayraghavan, 2003; Yao et al., 2008).
In rice, the C-class function is subfunctionalized by OsMADS3 and MADSS8, two homologs of AtAGAMOUS (AG) (Yamaguchi et al., 2006). In contrast with the case of Arabidopsis, in which the C-class gene AG mediates the specification of stamen and carpel identities, the control of floral meristem determinacy, and the inhibition of A-function gene activity, OsMADS3 plays a predominant role in stamen specification, whereas OsMADSS8 plays roles in the establishment of floral meristem determinacy and carpel development in rice (Yamaguchi and Hirano, 2006; Yamaguchi et al., 2006). Furthermore, the YABBY family gene DROOPING LEAF (DL) is required for carpel specification in rice (Yamaguchi et al., 2004).

A D-class gene of Arabidopsis, SEEDSTICK, specifies ovule identity redundantly with the C-class genes AG, SHATTER-PROOF1 (SHP1), and SHP2 (Pinyopich et al., 2003). In rice, one member of the D-class genes, MADDS13, but not the other member, MADDS21, is required for ovule identity specification, although redundant roles of the C-class genes MADDS3 and MADSS8 in ovule development are unknown (Dreni et al., 2007). Also in the rice genome, MADDS14, MADDS15, and MADDS18 are classified as AP1/FRUITFULL (FUL)-like (or A-class) genes, and LEAFY HULL STERILE1 (LHS1/MADDS1), MADDS5, MADDS7 (also called MADDS45), MADDS8 (also called MADDS24), and MADDS34 are classified as SEPALLATA-like (SEP-like; or E-class) genes, although most of the developmental functions of these genes, except for LHS1, have not been genetically proven (Jeon et al., 2000; Malcomber and Kellogg, 2005; Kater et al., 2006; Yamaguchi and Hirano, 2006).

In this study, we describe a novel rice mutant, mosaic floral organs1 (mfo1), which has defects in several floral organ identities, including the palea identity, and in floral meristem determinacy. We reveal that MFO1 is the AGAMOUS-LIKE6 (AGL6)-like MADDS box gene MADDS6 (Moon et al., 1999; Favaro et al., 2002). AGL6-like genes, which encode typical MIKC-type MADDS box proteins expressed in floral tissues, have been identified in many plant species for almost two decades and are regarded as a sister clade of SEP-like genes on the basis of phylogenetic analysis (Ma et al., 1991; Zahn et al., 2005). However, in contrast with the AP1/FUL-like, B-, C-, and D-class and SEP-like genes, little is known about the developmental function of AGL6-like genes, even in Arabidopsis, due to the lack of loss-of-function mutants in any plant species except for a recent report in petunia (Rijpkema et al., 2009). Our study revealed that the loss of AGL6-like function in rice resulted in aberrant floral morphology, demonstrating its pivotal role in floral development. We also show that the simultaneous knockdown of AGL6-like genes and the SEP-like gene LHS1 caused a loss of spikelet meristem determinacy. We propose that AGL6-like genes are required for the regulation of specification and development in rice floral organs and for the control of floral meristem fate.

**RESULTS**

We identified two recessive alleles, mfo1-1 and mfo1-2, of the MFO1 locus. Because the two allelic mutants show similar phenotypes, mainly mfo1-1 is described below.

**The mfo1 Mutant Shows Defects in Palea Identity**

The mfo1-1 mutant showed no abnormalities in the vegetative phase, but its florets were abnormal and almost sterile. The florets of mfo1-1 frequently had a slightly enlarged palea and were half open (Figures 1A and 1B). The half-open florets of mfo1-1 were likely caused by a lack of interlocking between the lemma and palea (Figures 1C and 1D). Further analysis revealed that the identity of the palea was poorly established. The lateral margin of the wild-type palea (marginal region of palea [mrp]) is membranous and has a smooth surface, whereas the mfo1-1 palea lacked the mrp and had a rough surface like that of the wild-type lemma (Figures 1E and 1F). Anatomical observation of the interlocking region revealed that large palisade-like cells, which normally develop on the adaxial surface of lemma, were formed on the adaxial surface of the mfo1-1 palea as well as on the lemma surface (Figures 1G and 1H). The number of vascular bundles increased from the normal three in the wild type to five in the mfo1-1 palea, the same number as in the lemma (Figures 1C and 1D). The margin of the mfo1-1 palea curled inside, similar to that of the wild-type lemma (Figure 1H). These morphological characteristics of the palea suggest that the mfo1-1 palea has acquired lemma identity, at least partially.

To further confirm the acquisition of lemma identity by mfo1-1 palea, we analyzed the expression of the DL gene that is expressed in lemma but not in the wild-type palea (Yamaguchi et al., 2004). DL was found to be weakly expressed in the mfo1-1 palea (Figure 1I). Thus, we conclude that the palea of mfo1-1 acquired partial lemma identity and that MFO1 is involved in the specification of palea identity.

**Defects in the Specification of Inner Floral Organ Identities in mfo1**

We also found severe phenotypes in the inner floral organs of mfo1-1. The wild-type rice floral organs consist of two lodicules in whorl 2, six stamens in whorl 3, and one pistil in whorl 4 (Figures 2A and 2B). However, the whorl structure of mfo1-1 flowers was severely disordered, and the floral organ identities were abnormal (Figures 2C and 2D).

Three types of floral organs were observed in the region corresponding to whorls 2 and 3 of the wild type (Table 1). Glume-like organs (ectopic glumes; Figures 2E and 2F), elongated lodicules (Figure 2H), and normal-shaped stamens (Figure 2I) were formed, but no normal lodicule was observed. Two to four ectopic glumes surrounded the center of the flower (Figures 2C and 2D). The ectopic glumes were hard and green, with a vascular bundle in the center, and had a rough surface with trichomes on the abaxial side (Figures 2E and 2F). Occasionally, the ectopic glumes grew as large in size as the normal palea (Figure 2G). The elongated lodicules were similar to the ectopic glumes in gross morphology but were soft and white (Figure 2H).

We performed RT-PCR analysis to compare the expression patterns of MADDS box genes in normal lodicules of the wild type and the elongated lodicules of mfo1-1 mutant plants (Figure 2M). In the wild-type lodicules, three B-class genes (MADDS2, MADDS4, and SPW1) were expressed, but two AP1/FUL-like genes (MADDS14 and MADDS15), two C-class genes (MADDS3 and
MADS58), and one SEP-like gene (LHS1) were not expressed. In mfo1-1 elongated lodicules, these AP1/FUL-like, C-class, and SEP-like genes were expressed in addition to the B-class genes, suggesting that this organ acquired a mosaic organ identity. The mfo1-1 stamens were apparently normal, although the number of stamens in a floret was significantly reduced (0 to 3) (Table 1). In addition, three types of higher-order mosaic floral organs were observed (Table 1): an ectopic glume with elongated lodicule tissue (Figure 2J), an intermediate organ of an elongated lodicule and stamen (Figure 2K), and a glume-lodicule-stamen mosaic organ (Figure 2L). mfo1-1 also displayed defects in whorl 4; the embryo sac rarely developed inside the abnormally fused carpels (Figure 2D). These results suggest that MFO1 is required for specification of the identities of the lodicule and ovule as well as for normal patterning of the inner floral organs.

**MFO1 Is Required for the Control of Floral Meristem Fate**

Figure 1. Rice mfo1-1 Mutant Shows Defects in Palea Identity.

(A) and (B) Spikelet of the wild type (A) and mfo1-1 (B). Palea is larger than lemma in mfo1-1.

(C) and (D) Cross sections of wild-type (C) and mfo1-1 (D) spikelet. Arrowheads indicate vascular bundles.

(E) and (F) Scanning electron micrograph of wild-type (E) and mfo1-1 (F) palea. Arrowhead indicates marginal region of palea (mrp) (E). In mfo1-1, no mrp was observed (arrowhead in [F]).

(G) and (H) Cross sections of wild-type (G) and mfo1-1 (H) palea edge. Edges of lemma and palea are hooked in the wild type (G), but there is no interlock with lemma in mfo1-1 (H).

(I) Relative expression levels of DL in lemma, palea, and abnormal palea of mfo1-1 and mfo1-2 compared with those of the wild type. Total RNA was pooled from ~10 pieces of tissue of each genetic background. The samples were quantified by real-time RT-PCR using UBQ mRNA as a reference. Error bars indicate the SE for three PCR reaction/detection replicates in a single experiment.

a-pa, abnormal palea; le, lemma; mrp, marginal region of palea; pa, palea. Bars = 2 mm in (A) and (B), 400 μm in (C) and (D), 500 μm in (E) and (F), and 100 μm in (G) and (H).

Abaxial (to the rachis axis) edge of the receptacle (or the rachilla, the basic axis-like region of the rice floret), and the palea was formed at a slightly higher position on the top abaxial edge of the same receptacle, just inside the lemma (Figures 3A and 3B). In mfo1-1, the axis-like region was further elongated from the original lemma to the abnormal palea (Figure 3C). This region appeared to be formed by the elongation of the receptacle, and it sometimes subtended the ectopic glumes (Figure 3D). Elongation of the axis-like structure with the ectopic glume hindered the initiation of inner floral organ development, implying that the establishment of floral meristem identity was retarded.

A new axis-like structure was also observed, again in whorl 4, and new abaxial or mosaic floral organs were subsequently formed on its top (Figure 3E). This axis-like structure possessed vascular bundles, suggesting further elongation of the receptacle (Figure 3F). A glume-like structure was often formed in the center of the mfo1-1 floret, and the central glume-like organ covered organs of unknown identity or abnormal carpels (Figures 3E, 3G, and 3H). The abnormal carpel, frequently lacking stigmatic papillae, often developed reiteratively, but it sometimes
possessed abnormal ovule-like structures (Figures 3G to 3I). These results suggest that defects in floral meristem determination resulted in the repetitive formation of abnormal carpels. Occasionally, a new immature spikelet that possessed a pair of rudimentary glumes, a pair of empty glumes, lemma, palea, and a floral meristem emerged from the top of mfo1-1 florets (Figure 3J), indicating the recall of the spikelet meristem identity.

Two AP1/FUL-like MADS box genes, MADS14 and MADS15, are upregulated in the early stages of panicle/spikelet development (Furutani et al., 2006). Therefore, we compared the expression patterns of MADS14 and MADS15 in the abnormal carpel of mfo1-1 with those in the normal carpel of the wild type. We also examined the expression patterns of C-class (MADS3 and MADS58) and D-class (MADS13 and MADS21) genes and DL that specifies carpel identity (Figure 3K). In the abnormal carpels of mfo1-1, in addition to the C- and D-class MADS box and DL genes, AP1/FUL-like MADS box genes, which were not expressed in wild-type carpels, were also expressed. These results suggest that ectopic expression of AP1/FUL-like genes in whorl 4 leads to ectopic spikelet formation in the mfo1-1 floret.
MFO1 Is the AGL6-Like MADS Box Gene Os MADS6

To understand its molecular function, we isolated the MFO1 gene by map-based cloning. Using restriction fragment length polymorphism and cleaved- amplified polymorphic sequence markers, MFO1 was mapped to the long arm of chromosome 2 between the markers P0135D07-2 (on the BAC clone OSJN88b0005A04; accession number AP005775) and OJ1212_A08-3 (on the BAC clone OJ1212A08; accession number AP004255) (see Supplemental Table 1 online). In this region spanning four BAC/P1-derived artificial chromosome clones, a floral MADS box gene, MADS6, was annotated. Sequence analysis identified one base substitution in the mfo1-1 allele. This mutation causes an amino acid substitution from the conserved Arg to His (R24H) at the 24th codon in the MFO1 protein (GFP)-tagged allele. This mutation causes an amino acid substitution from the conserved Arg to His (R24H) at the 24th codon in the MFO1 protein.

This arrangement was similar to the position of mfo1-1 ectopic glumes. Some mfo1-2 lodicules possessed a glume-like green region (Figure 5F). In whorl 3, the number of stamens was frequently decreased (Table 1). In addition, lodicules-stamen mosaic floral organs (Figure 5G) or fused stamens (Figure 5C) were formed. Although the mfo1-2 carpel was apparently normal, ovule development was sometimes disturbed. In the abnormal ovule, the outer integument showed outgrowth, and the embryo sac did not develop and was replaced by an abnormally proliferated nucellus (Figures 5C, 5H, and 5I). These observations are consistent with the low seed fertility of mfo1. In summary, the moderate phenotypes of mfo1-2 indicate that it is a partial loss-of-function allele. We also generated 20 Os MADS6 RNA interference (RNAi) transgenic plants, and the phenotypes of 16 RNAi lines were similar to those of mfo1-2, further confirming that rice MADS6 is the MFO1 gene (Figure 5J).

Hereafter, we refer to this gene as MFO1 based on its phenotypes.

Phylogenetic Analysis of Rice AGL6-Like MADS Box Genes

To gain insight into the function of MFO1, we generated a phylogenetic tree using MADS box protein sequences from rice and other plants (see Supplemental Table 2 and Supplemental Data Set 1 online). MFO1 was grouped into the AGL6-like subfamily together with Arabidopsis AGL6 and AGL13 (Figure 4B). Many AGL6-like genes have been reported in other plants, including monocots, dicots, and gymnosperms. Some of these genes have been reported to be expressed in floral organs. In rice, MADS17 also encodes an AGL6-like protein that shows strong similarity to MFO1. Among all classes of MADS box proteins, the AGL6-like subfamily of proteins is most similar to the SEP-like proteins.

We found two well-conserved sites in the C-terminal regions of AGL6-like proteins (Figure 4C). The AGL6-I motif is located in the middle part of the C-terminal region, with the 10–amino acid sequence DCEPTLQIGY forming a consensus. This motif is highly conserved in monocots. In MFO1, this motif is well conserved, although the second Cys is substituted with Ser. The AGL6-II motif is at the end of the C-terminal region, with the 10–amino acid sequence ENNFMLGWVL as a conserved motif in monocots, dicots, and gymnosperms. Some of these motifs are conserved in rice.
residues at the end of this motif. The AGL6-I and AGL6-II motifs are similar to the previously reported SEP I and SEP II motifs conserved near the C termini of SEP-like proteins (Figure 4C; Zahn et al., 2005).

Although these motifs are suggested to be transcriptional activation motifs, no molecular evidence has been found to support this suggestion (Zahn et al., 2005). Therefore, we fused these motifs with the DNA binding domain of the yeast GAL4 protein (GAL4-BD) and examined their transcriptional activities in yeast. When the C-terminal region of MFO1 (42 residues) or MADS17 (41 residues) containing the region from the AGL6-I to the AGL6-II motif was fused with GAL4-BD, both fusion proteins showed transcriptional activation using LacZ and HIS3 reporter genes in yeast (Figure 6). These results suggest that the C termini of AGL6-like proteins mediate transcriptional activation. To further localize the transcriptional activation domain, we generated deletion constructs derived from the C terminus of MFO1 and examined their activity in yeast. In these assays, the fusion proteins containing the AGL6-II motif showed strong transcriptional activation. The fusion proteins containing the AGL6-I motif and/or the intervening region, but not the AGL6-II motif, also showed moderate transcriptional activation. These results indicate that the C-terminal region of AGL6-like proteins spanning from the AGL6-I motif to the AGL6-II motif is a potential transcriptional activation domain, and the AGL6-II motif mediates particularly strong transcriptional activation.

Figure 3. mfo1-1 Mutant Shows Defects in Floral Meristem Fate.

(A) and (C) Scanning electron micrograph of wild-type (A) and mfo1-1 (C) spikelet. Lemmas and palea were removed. Arrows and arrowheads indicate the base position of lemma and palea/abnormal palea, respectively. Brackets show elongated portions of receptacle. (B) and (D) Longitudinal sections of wild-type (B) and mfo1-1 (D) spikelet. Arrowheads indicate vascular bundles. Arrow indicates ectopic glume. (E) Centre of mfo1-1 flower. Arrow indicates an organ with unknown identity. Arrowhead indicates elongated receptacle observed in the inner whorl. (F) Cross section of an elongated receptacle of mfo1-1 flower at the position comparable to the arrowhead in (E). Arrowhead indicates vascular bundles. (G) Reiterated abnormal carpels of mfo1-1. Arrowhead indicates a small ectopic glume that covers abnormal carpels. (H) Scanning electron micrograph of reiterated abnormal carpels (same sample as (G)). Arrowhead indicates a small ectopic glume. (I) Cross section of an abnormal carpel. Arrowhead indicates abnormal ovule-like structure. (J) Scanning electron micrograph of mfo1-1 whorl 4. Arrowheads indicate new spikelets. Arrow indicates a small ectopic glume. (K) RT-PCR analysis of DL and MADS box genes expression in wild-type carpels and mfo1-1 abnormal carpels. APT1 was used as a control. PCR cycle number was 30.

ac, abnormal carpel; a-pa, abnormal palea; eg, empty glume; le, lemma; lo, lodicule; pa, palea; pi, pistil; rc, receptacle; st, stamen. Bars = 142 μm in (A) and (C), 400 μm in (B) and (D), 1 mm in (E) and (G), 200 μm in (F), (H), and (I), and 333 μm in (J).
Figure 4. Structure and Phylogenetic Analysis of the MFO1 Gene.

(A) MFO1/MADS6 is composed of eight exons and seven introns. Boxes indicate exons, and lines between boxes indicate introns. mfo1-1 has a single amino acid substitution in the MADS domain. Tos17 retrotransposon is inserted in the 8th exon in mfo1-2.

(B) Phylogenetic tree of MADS box proteins. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar et al., 2004). Phylogenetic tree was

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Expression Patterns of Rice AGL6-Like Genes

The expression pattern of MFO1/MADS6 has been reported (Moon et al., 1999; Favaro et al., 2002; Lee et al., 2003). However, previous studies did not examine the detailed expression pattern of MFO1 in the palea, lodicule, and pistil, which morphologically were observed in mfo1, or lemma or at early stages of panicle development. To further understand the function of MFO1, we used RT-PCR to examine its expression pattern spatially (in the empty glume, lemma, palea, lodicule, stamen, and pistil of the mature spikelet) and temporally (during different stages of panicle development). We also examined the expression pattern of MADS17.

No MFO1 expression was detected in young panicles of 0.5 mm, but its expression initiated at the 1-mm stage and was maintained at the 3- and 5-mm stages (Figure 7A). The 0.5-mm young panicle differentiates primary branches, but no floral organs. The empty glume, lemma, and palea begin to differentiate at the 1-mm stage (stage In6/Sp2–4), and primordia of the inner floral organs (lodicule, stamen, and pistil) differentiate thereafter (stage In7/Sp5–6) (Ikeda et al., 2004; Itoh et al., 2005). In mature spikelets, MFO1 was expressed in palea, lodicule, and pistil. Faint expression was observed in empty glumes and stamens, but no expression was detected in the lemma (Figure 7B). Considering that the palea possesses the specialized marginal regions, these results suggest that MFO1 is required to specify palea identity.

RT-PCR analysis revealed that faint expression of MADS17 was detected at the 0.5-mm stage and established after the 1-mm stage (Figure 7A). MADS17 was expressed in palea, lodicule, and pistil and weakly in empty glumes and stamens, in addition to MFO1. However, in contrast with MFO1, MADS17 was expressed in the lemma (Figure 7B). To further investigate the expression patterns of rice AGL6-like genes, we performed in situ hybridization analysis. At stage Sp3, when the lemma primordium initiates, MFO1 expression was detected in the floral meristem but not in the lemma, empty glumes, or rudimentary glumes (Figure 7C). At stage Sp5–6, when the lodicule and stamen primordia initiate, MFO1 was expressed at the palea primordium but not at the incipient stamen primordium (Figure 7D). At stage Sp7, when the carpel primordium initiates, MFO1 was strongly expressed in the palea, lodicule, and floral meristem/carpel primordium but not in stamen primordia (Figure 7E). Later, at stage Sp8, when the ovule and pollen initiate, MFO1 was expressed in the receptacle, lodicule, and integument but not in the carpel (Figure 7F). Notably, MFO1 was expressed in the mrp (Figure 7G). Furthermore, the expression pattern of MFO1 in the mrp, lodicule, and pistil of the immature floret was confirmed by observation of gMFO1:GFP plants (Figure 7H). At stages Sp3–4, when the lemma and palea initiate, MADS17 was also expressed in the floral meristem (Figure 7I). At stage Sp7, MADS17 was expressed in the lodicule and palea. In addition, MADS17 was expressed in the lemma, in which MFO1 was not expressed (Figure 7J). At stage Sp8, MADS17 was expressed in the receptacle, lodicule, and ovule but not in the mrp (Figures 7K and 7L). In total, MFO1 was expressed in areas in which morphological abnormalities were observed, and MADS17 expression generally overlapped that of MFO1.

Genetic Interaction between MFO1 and MADS17

To further clarify the AGL6-like functions, we investigated the genetic interaction between MFO1 and MADS17. First, we examined the transcription levels of MFO1 and MADS17 in mfo1-2 and MFO1 RNAi (MFO1i) spikelets by quantitative RT-PCR. In the MFO1i plants examined, the MFO1 transcript level was more severely downregulated than in mfo1-2, even though the phenotypes of these RNAi plants were comparable to those of mfo1-2 (Figures 5A and 8A, Table 2). Interestingly, the MADS17 transcript levels in both mfo1-2 and MFO1i plants were slightly higher than in the wild type. This observation raises the possibility that upregulated MADS17 masks the effect of the MFO1 knockdown. Therefore, to examine the potential effect of MADS17 on floral development, we generated 16 MADS17 RNAi (OM17i) plants in which the expression of MADS17 was specifically downregulated (Figure 8A). In contrast with the MFO1i lines, which showed phenotypes similar to those of mfo1-2, none of the OM17i plants showed any alteration in floral morphology (Figures 8A and 8C, Table 2).

We then generated various combinations of MFO1 and MADS17 knockdown plants in the wild-type and mfo1-2 backgrounds (Table 2). When introduced into mfo1-2, MFO1i enhanced the phenotypes of mfo1-2 in 15 of the 18 transgenic plants. Most of the whorl-2 organs in these plants were converted into elongated lodicules with a green region and sometimes subtended ectopic glume-like organs (Figure 8D). In MFO1i mfo1-2, the MFO1 transcript levels were lower than in mfo1-2 but comparable to those in MFO1i (Figure 8B), suggesting that translational inhibition may have enhanced the phenotypes compared with the MFO1i plants (Brodersen et al., 2008). By contrast, the introduction of OM17i caused no significant enhancement of the mfo1-2 phenotypes in 10 transgenic plants (Figure 8E). We also generated 20 double RNAi (MFO1i+OM17i) plants in the wild-type background. Unlike the MFO1i lines, in which the expression of MFO1 but not MADS17 is specifically downregulated, the expression of both MFO1 and MADS17 was simultaneously downregulated in MFO1i+OM17i plants (Figure 8A). Whereas the phenotypes of 14 MFO1i+OM17i plants were comparable with those of MFO1i plants, two MFO1i+OM17i plants showed moderate phenotypes comparable to that of MFO1i mfo1-2 (Figure 8F).

Figure 4. (continued).
These results suggest that MADS17 plays minor but redundant roles with MFO1 in floral development and that its minor functions may not be observed unless the function of MFO1 is severely impaired. To further confirm the role of MADS17, we introduced the MFO1i+OM17i double-RNAi cassette into mfo1-2. In these mfo1-2-based double-RNAi plants, although the MFO1 expression levels were comparable to those in the MFO1i mfo1-2 plants, the MADS17 expression levels were significantly lower than those in MFO1i mfo1-2 plants (Figure 8B). In contrast with MFO1i mfo1-2 plants, 11 out of the 18 MFO1i +OM17i mfo1-2 transgenic plants showed strongly enhanced phenotypes compared with that of mfo1-2, somewhat similar to that of mfo1-1: elongated lodicules were predominantly replaced by ectopic glumes, and ectopic spikelets developed at the center of the floret (Figures 8G and 8H, Table 2). Furthermore, an extra axis-like structure and mosaic floral organs similar to those of mfo1-1 also developed in these florets. Taken together, these results clearly indicate that MADS17 also has minor but redundant AGL6-like functions, together with MFO1.

MFO1 and LHS1 Function Redundantly in Regulating Floral Meristem Fate

Because these results suggested a function for MFO1 in regulating meristem fate, we examined the genetic interaction of MFO1 with LHS1, which has also been suggested function in floral meristem fate.
A Tos17 insertion mutant line, NF1019, was reported to harbor a weak *lhs1* allele. In this line, a Tos17 insertion in the seventh exon caused alternative splicing, resulting in a deletion of 38 amino acids in the C-terminal domain and conferred a mild phenotype (Agrawal et al., 2005). Here, we refer to the NF1019 allele as *lhs1*-2, whereas the *lhs1* allele reported by Jeon et al. is referred to as *lhs1*-1 (Jeon et al., 2000). Although Agrawal et al. reported that >50% of spikelets in *lhs1*-2 formed extra spikelets with elongated pedicels at the center of the florets, at our experimental farm and greenhouse of Hokuriku Research Center, most of the *lhs1*-2 spikelets did not show such ectopic spikelets, but did show variable mild phenotypes with reduced lemma, reduced mrp-less palea, and two to four mrp-like or glume-like organs surrounding the normal floral organs (Figure 9A).

We generated 19 MFO1+OM17i double-RNAi lines in the *lhs1*-2 mutant background. Among the transgenic plants, 10 showed severe phenotypes, whereas nine plants showed almost the same phenotype as the single *lhs1*-2 mutant. In the severe transgenic lines, >90% of the spikelets lacked the inner floral organs and ~30% of the spikelets possessed ectopic spikelets with elongated pedicels at the center of the florets, at our experimental farm and greenhouse of Hokuriku Research Center, most of the *lhs1*-2 spikelets did not show such ectopic spikelets, but did show variable mild phenotypes with reduced lemma, reduced mrp-less palea, and two to four mrp-like or glume-like organs surrounding the normal floral organs (Figure 9A).

We obtained 19 MFO1+OM17i double-RNAi lines in the *lhs1*-2 mutant background. Among the transgenic plants, 10 showed severe phenotypes, whereas nine plants showed almost the same phenotype as the single *lhs1*-2 mutant. In the severe transgenic lines, >90% of the spikelets lacked the inner floral organs and ~30% of the spikelets possessed ectopic spikelets with elongated pedicels (Figures 9B and 9C, Table 3). We also obtained the *mfo1*-2 *lhs1*-1 double mutant. This mutant showed variable but severe spikelet phenotypes similar to those of MFO1+OM17i *lhs1*-2 and rarely showed the mild phenotype observed in the *lhs1*-2 single mutant. The *mfo1*-2 *lhs1*-2 double mutant spikelets could be classified into three types: (1) one or a few sequential extra spikelet(s) lacking inner floral organs (Figure 9D), (2) extra glume-like organs with no inner floral organs (Figure 9E), and (3) abnormal lemma and palea lacking floral organs (Figure 9F).

Taking account of the phenotypes of *mfo1*-1 and MFO1+OM17i *mfo1*-2 in terms of ectopic spikelet formation, the phenotypes of *mfo1*-2 *lhs1*-2 spikelets resemble enhanced versions of the phenotypes of MFO1+OM17i and *mfo1*-2. Wild-type spikelets initiate stamen primordia at stage Sp6, when the margins of the lemma and palea primordia overlap (Figure 9G) (Ikeda et al., 2004). By contrast, the severe spikelets of the *mfo1*-2 *lhs1*-2 mutant did not form stamen primordia but possessed enlarged or divided meristematic tissues at the center (Figures 9H and 9I). These results indicate that the control of meristem fate is abnormal in the *mfo1*-2 * lhs1*-2 double mutant. More specifically, the enhanced severe phenotype compared with those of the *lhs1*-2 and *mfo1*-2 single mutants indicate that spikelet meristem determinacy and the transition to the floral meristem, which are redundantly regulated by MFO1 and LHS1, are likely impaired in the double mutant.

**DISCUSSION**

**AGL6-Like Genes Regulate Floral Development in Rice**

The *Arabidopsis* genome contains two AGL6-like genes, *AGL6* and *AGL13*. *AGL6* is preferentially expressed in floral buds, whereas *AGL13* is expressed mainly in ovules (Ma et al., 1991; Rounsley et al., 1995). The AGL6 protein interacts with a C-class MADS box protein, AG, and further yeast two-hybrid studies in several plant species revealed that AGL6-like proteins interact with AP1/FUL-like, B-class, D-class, and SEP-like MADS box proteins (Fan et al., 1997; Moon et al., 1999; Favoro et al., 2002; Cooper et al., 2003; Hsu et al., 2003; de Folter et al., 2005). However, ectopic expression of AGL6-like genes from orchid (*Oncidium OMADS1*), Norway spruce (*Picea abies DAL1*), or hyacinth (*Hyacinthus orientalis* AGL6) in transgenic Arabidopsis resulted in early flowering, suggesting that they have roles in flowering, which is consistent with their endogenous expression patterns (Hsu et al., 2003; Carlsbecker et al., 2004; Fan et al., 2007). Despite this information, the developmental roles of AGL6-like genes, especially in flower development, remain unclear.

We described the unequally redundant floral regulators *MFO1* and MADS17 as AGL6-like genes of rice. We showed that an AGL6-like MADS box gene, *MFO1*, is a key regulator of organ identity and meristem fate in rice. Another rice AGL6-like gene, MADS17, seems to play minor but redundant roles with *MFO1* in floral development. The upregulation of MADS17 in the *MFO1*...
knockdown plants suggested that a potential genetic interaction exists between these two genes. Downregulation of MFO1 may have caused a feed-forward regulation of MADS17 expression. Alternatively, the upregulation of MADS17 may result from increased numbers of elongated lodicules and/or abnormal palea, in which MADS17 is expressed.

The mfo1-1 allele showed a more severe phenotype than mfo1-2, but it is not clear whether mfo1-1 is a null or a weak dominant-negative allele. mfo1-1 contains an R24H missense mutation. The lhs1-1 allele also possesses a missense mutation at the 24th Arg (R24C) next to a G27D mutation and is suggested to be a weak dominant-negative allele (Jeon et al., 2000). The conserved 24th Arg in the α-helix of the MADS domain is involved in both DNA contact and the dimerization of MADS proteins (Pellegrini et al., 1995; Han et al., 2003). In the MFO1i and MFO1i mfo1-2 plants, the MFO1 expression levels were significantly lower, although they showed much milder phenotypes than mfo1-1. Therefore, the R24H mutation in mfo1-1 may also have caused a dominant-negative effect as was suggested for lhs1-1. If this were the case, the MFO1R24H mutant protein would most

Figure 7. Expression Pattern of MFO1 and MADS17.

(A) RT-PCR analysis of the two AGL6-like MADS box genes in different panicle developmental stages. Total RNA was isolated from leaves (Lf), young panicle whose length was <0.5, ~1, ~3, and ~5 mm. Fifty nanograms of total RNA was used to analyze MFO1 and ACTIN1 (control), and 150 ng of total RNA was used to analyze MADS17. PCR cycle number was 30 for MFO1 and MADS17, and 25 for ACTIN1.

(B) RT-PCR analysis of MFO1 and MADS17 expression in various floral organs of the wild type at heading date stage. Total RNA was isolated from each floral organ, and 30 ng RNA was analyzed. PCR cycle number was 30 for MFO1 and MADS17, and 27 for ACTIN1 (control).

(C) In situ hybridization of MFO1 transcripts in the Sp3 spikelet. Black arrowhead indicates primordium of palea. MFO1 was not expressed at the incipient stamen primordia (asterisks).

(D) In situ hybridization of MFO1 transcripts in the Sp5-6 spikelet. Black arrowhead indicates expression in carpel primordium. The transcripts were also detected in palea and lodicule but not in stamen.

(E) In situ hybridization of MFO1 transcripts in the Sp7 spikelet. Black arrowhead indicates expression in carpel primordium. The transcripts were also detected in palea and lodicule but not in stamen.

(F) In situ hybridization of MFO1 transcripts in the Sp8 spikelet. Black arrowhead indicates the expression in integument within the carpel. The transcripts were also detected in lodicule and receptacle but not in carpel.

(G) In situ hybridization of MFO1 transcripts in a cross section of the Sp8 spikelet. Black arrowheads indicate the marginal regions of palea.

(H) An immature floret of gMFO1:GFP plant. Fluorescence was observed in mrp, lodicule, and pistil.

(I) In situ hybridization of MADS17 transcripts in the Sp3-4 spikelet.

(J) In situ hybridization of MADS17 transcripts in the Sp7 spikelet. Arrow and arrowhead indicate part of lemma and palea, respectively, where MADS17 transcripts were detected. The transcripts were also detected in lodicule.

(K) In situ hybridization of MADS17 transcripts in the Sp8 spikelet. Arrowhead indicates ovule. The transcripts were also detected in lodicule and receptacle.

(L) In situ hybridization of MADS17 transcripts in the Sp8 spikelet in cross section. The transcripts were not detected even in the mrp.

eg, empty glume; fm, floral meristem; le, lemma; lo, lodicule; mrp, marginal region of palea; pa, palea; pi, pistil; rc, receptacle; rg, rudimentary glume; st, stamen. Bars = 100 μm in (C) to (E), (I), and (J), 400 μm in (F) and (K), and 200 μm in (G) and (L).
probably disturb its paralogous protein MADS17. The phenotypic severity common to mfo1-1 and MFO1i+OM17i mfo1-2 suggests this idea.

Roles of MFO1 in Floral Organ Specification, Development, and Pattern Formation

In mfo1 florets, various abnormalities were observed. These defects were observed in the region where MFO1 is expressed in the wild type. MFO1 is required for the identity specification and development of these floral organs, possibly in combination with various interaction partner proteins (Figure 10). The phenotype of the mfo1 abnormal palea suggested that it has acquired partial lemma identity. Because the loss of mrp from the palea was also reported in knockout/knockdown plants of LHS1, whose protein product interacts with MFO1 (Moon et al., 1999; Jeon et al., 2000; Prasad et al., 2005), the normal development of the mrp may require the activity of a protein complex containing both MFO1 and LHS1. However, in contrast with mfo1, lhs1 mutants frequently possess membranous mrp-like organs between the abnormal palea and the inner floral organs (Jeon et al., 2000; Agrawal et al., 2005; Chen et al., 2006), suggesting that the specification of mrp identity does not require LHS1 activity.

mfo1 florets showed abnormalities mainly in whorls 2 and 4. Rice AGL6-like genes are expressed in these whorls of the wild type, and knockdown of AGL6-like function causes the misexpression of several classes of MADS box genes, resulting in various levels of mosaic floral organs, possibly via the formation of ectopic MADS box protein complexes. These results suggest a suppressive effect of MFO1 on the expression of AP1/FUL-like, C-class, and SEP-like genes in whorl 2 and AP1/FUL-like genes in whorl 4, although the C-terminally conserved motifs of AGL6-like proteins showed transactivation activity in our yeast experiments. Whereas Arabidopsis SEP3, which also shows transactivation activity in yeast, is involved in the upregulation of several MADS box genes (AP1, AP3, AG, SHP1, and SHP2), it has also been suggested to be involved in the downregulation of other sets of MADS box genes (AGL24, SOC1, and SVP),
whether there were fewer stamens per floret. At present, it is not clear whether the formation of ectopic organs larger than the original organs results in the perturbation of whorl structure.

The expression pattern and mutant phenotype (reiteration of carpel and ectopic spikelet formation) of MFO1 suggest that it is associated with meristem fate. Reiteration of the carpel has also been reported in the osmads3 and osmads13 mutants (Yamaguchi et al., 2006; Dreji et al., 2007), and ectopic spikelet formation occurs in *lhs1* (Jeon et al., 2000; Agrawal et al., 2005). Therefore, the reiteration of ectopic carpels or ectopic spikelets possibly as a part of protein complexes (Kaufmann et al., 2009; Liu et al., 2009), MFO1 may have similar dual functions depending on the composition of the multiprotein complexes in which it participates. Although the expression of an AP1/FUL-like gene, MADS15 (also called RAP1A), in the lodicule at early developmental stages was reported (Kyoizuka et al., 2000), our RT-PCR analysis revealed that neither MADS14 nor MADS15 is expressed in mature wild-type lodicules. Taking account of the roles of C-class genes in lodicule development (Yamaguchi et al., 2006), the suppression of AP1/FUL-like and C-class genes in whorl 2 would be necessary for the normal development of the lodicule. Similarly, the suppression of AP1/FUL-like genes may be necessary for the normal development of ovule and floral meristem determinacy in whorl 4.

Considering its overlapping expression in integuments and its protein–protein interaction patterns, a protein complex containing MFO1 and MADS13 is likely to regulate correct integument development (Favaro et al., 2002). As reported in several species, including rice, this abnormal integument development would disturb normal ovule development (Robinson-Beers et al., 1992; Gaiser et al., 1995; Baker et al., 1997; Yamaki et al., 2005). In contrast with the defect in whorls 2 and 4, our results indicate that MFO1 is not necessary for stamen identity specification, consistent with its expression pattern.

MFO1 is also thought to be necessary for floral patterning, as suggested by the perturbed whorl structure in *mfo1-1*. Also in the *mfo1-2* floret, abnormal lodicules were ectopically formed and there were fewer stamens per floret. At present, it is not clear whether MFO1 has a direct role in the formation of the whorl structure through establishing the boundaries between whorls or whether the formation of ectopic organs larger than the original organs results in the perturbation of whorl structure.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency of Plants Showing Abnormal Phenotype</th>
<th>Summary of Notable Phenotypes in Inner Floral Whors</th>
</tr>
</thead>
<tbody>
<tr>
<td>MADS17 RNAi (OM17i)</td>
<td>16/16</td>
<td>No phenotype.</td>
</tr>
<tr>
<td>MFO1 RNAi (MFO1i)</td>
<td>16/20</td>
<td>Mild. Elongated lodicules.</td>
</tr>
<tr>
<td>OM17i mfo1-2</td>
<td>10/20</td>
<td>Mild. Elongated lodicules.</td>
</tr>
<tr>
<td>MFO1i+OM17i</td>
<td>14/20</td>
<td>Intermediate. Elongated lodicules with green regions. Sometimes subtended ectopic glume-like organs.</td>
</tr>
<tr>
<td>mfo1-2</td>
<td>15/18</td>
<td>Intermediate. Elongated lodicules with a green region. Sometimes subtended ectopic glume-like organs.</td>
</tr>
<tr>
<td>MFO1i+OM17i mfo1-2</td>
<td>11/18</td>
<td>Severe. Ectopic glumes, ectopic spikelets, extra axis-like structure, and mosaic floral organs.</td>
</tr>
</tbody>
</table>

The numbers indicate the numbers of plants showing the phenotype per total numbers of that genotype.

**Figure 9.** MFO1 and LHS1 Redundantly Control Floral Meristem Fate.

(A) Spikelet of *lhs1-2*. Arrowheads indicate ectopic mrp-like organs. Note that inner floral organs were formed. Lemma and palea were removed. **(B)** and **(C)** Spikelet of MFO1i+OM17i *lhs1-2*. Arrowhead indicates the pedicel of an ectopic spikelet (B). Arrow indicates the ectopic glume-like organ (C). Note that no inner floral organ was formed in both (B) and (C). Lemma and palea were removed. **(D)** to **(F)** Spikelet of mfo1-2 *lhs1-2*. Arrowheads indicate the pedicels of sequential ectopic spikelets (D). Arrow indicates the ectopic glume-like organ (E). Note that no inner floral organ was formed in both (D) and (E). No organ was formed inside lemma and palea (F). Front-side halves of lemma and palea were removed. **(G)** to **(I)** Scanning electron micrograph of the wild-type (G) or mfo1-2 *lhs1-2* (H) and (I) spikelets at the stage Sp6. Arrowheads indicate meristematic tissues.
in the center of the mfo1 flower may be caused by defects in the formation of complexes of MFO1 with MADS3, MADS13, or LHS1.

The mfo1 flower also has an elongated receptacle-like structure bearing lemma-like organs. The occurrence of these structures in mfo1 suggests that the formation of inner floral organs requires a transition of the floral meristem fate specifically controlled by MFO1. This meristem control function seems to be redundant with other factor(s), because inner floral organs were finally formed inside the ectopic glumes in the mfo1 single mutant. The mutant phenotypes of a SEP-like gene, LHS1, suggest that it has a regulatory function in the rice floral meristem (Jeon et al., 2000; Agrawal et al., 2005; Prasad et al., 2005; Chen et al., 2006; Yamaguchi and Hirano, 2006), and a protein–protein interaction between MFO1/MADS6 and LHS1/MADS1 has been reported (Moon et al., 1999). Our double mutant analysis clearly revealed that the SEP-like gene LHS1 shares meristem functions with MFO1. As we described, the mfo1 lhs1 double mutants showed three types of severe meristem-related phenotypes. The type I phenotypes were likely caused by a defect in the transition from spikelet meristem to floral meristem, and the maintenance of spikelet meristem activity may have regenerated extra inner spikelets. In the case of the type II and III phenotypes, the spikelet meristem probably failed to convert into a floral meristem, and then the meristem activity was consumed. In the case of type II spikelets, some slight remaining spikelet meristem activity would regenerate extra mrp-like or glume-like organs.

In Arabidopsis, although many genes that control floral organ identities and patterning have been reported, the roles of AGL6-like genes are still unknown. Rijpkema et al. (2009) recently suggested redundant functions for the petunia AGL6-like gene Ph AGL6 and the SEP-like genes Ph FBP2 and Ph FBP5. Although a petunia agl6 single mutant (or RNAi transgenic plants for Ph AGL6) showed no morphological defects, the petunia agl6 fbp2 double mutant and agl6 fbp2 fbp5 triple mutants showed enhanced phenotypes compared with the fbp2 single mutant and fbp2 fbp5 double mutants, respectively. These results suggest a functional redundancy of Ph AGL6 with SEP-like genes. Interestingly, mutations in Ph AGL6 caused mild defects in stamen identity specification when combined with mutations in SEP-like genes. The discrepancy between the stamen phenotypes may be caused by different expression patterns in the stamen between rice and petunia because Ph AGL6 is expressed weakly in stamens, in contrast with the almost nonexistent expression of rice MFO1 in stamens. In rice, many genes homologous to Arabidopsis developmental regulators, such as APO1 (a rice UFO homolog; Ikeda et al., 2007), RFL (a rice LFY homolog; Rao et al., 2008), and FON1 (a rice CLV1 homolog; Suzaki et al., 2004), have been reported to have roles in floral development that are divergent from those of Arabidopsis genes. Therefore, the roles of MFO1 might also be divergent from those of AGL6 and AGL13 in Arabidopsis and their orthologs in other eudicots.

### Implications for the Identity of the Rice Palea

The identity of the rice palea is controversial (Schmidt and Ambrose, 1998; Kellogg, 2001; Nagasawa et al., 2003). In grass flowers, the palea is generally thought to have a different identity from the lemma; 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

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of Spikelets Examed</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>#18</td>
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<td>14 (41%)</td>
<td>18 (53%)</td>
<td>0 (0%)</td>
<td>32 (94%)</td>
</tr>
<tr>
<td>#17</td>
<td>24</td>
<td>6 (25%)</td>
<td>16 (67%)</td>
<td>2 (8%)</td>
<td>24 (100%)</td>
</tr>
<tr>
<td>#19</td>
<td>10</td>
<td>3 (30%)</td>
<td>7 (30%)</td>
<td>0 (0%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>#12</td>
<td>39</td>
<td>10 (26%)</td>
<td>20 (51%)</td>
<td>1 (3%)</td>
<td>31 (79%)</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>33 (31%)</td>
<td>61 (57%)</td>
<td>3 (3%)</td>
<td>97 (91%)</td>
</tr>
</tbody>
</table>

*Type I, regenerating one or a few sequential spikelets lacking inner floral organs. Type II, bearing extra glume-like organs but no inner floral organ formed. Type III, no organ formation inside the abnormal lemma and palea.

Table 3. Phenotypes of MFO1+OM17+lhs1-2 Transgenic Plants

- **Figure 10.** An Integrated Model for AGL6-Like Function in Rice Floret Development.

- Proved and proposed functions of AP1/FUL-like, B-, C-, D-class genes, LHS1, and DL are also integrated in this model. AP1/FUL-like genes, MADS14 and MADS15, are expressed in early stages of lodicule but not in later stages of lodicule (Kyozuka et al., 2000; this study).
Figure 10). This fate might be determined at the initiation of the spikelet axis (Kellogg, 2001). Nevertheless, the outer morphology of the rice palea resembles that of the lemma, implying that the palea is closely related to the lemma in rice. In the rice B-class mutant spw1 and MADS2 + MADS4 double-RNAi plants, lodicules were transformed into mrp-like organs, suggesting that these organs formed in whorl 2 may be equivalent to sepals and that the body of the rice palea (the portion of the palea other than the mrp) might have bract-like characteristics similar to those of the lemma (Nagasawa et al., 2003; Yao et al., 2008). Consistent with these hypotheses, the phenotypes of the mfo1 palea suggest that the rice palea might be a fused organ between the mrp and lemma-equivalent organ.

We suggest that the expression of MFO1 in the lateral margin of the palea primordia causes the formation of the mrp, possibly through interaction with SEP-like and/or AP1/FUL-like proteins (Figure 10). This fate might be determined at the initiation of the palea primordium, where the presumptive expression of MFO1 was observed. The lateral outgrowth of the abnormal mfo1 palea suggests that formation of the mrp inhibits this lateral growth and thus establishes the normal palea identity. By contrast, MFO1 is not required for the initiation and development of the lemma-like portion of the palea. The palealess1 (pal1) and retarded palea1 (rep1) mutants of rice, which show the loss/retardation of the body of the palea and the emergence/outgrowth of two leaf-like structures, support our hypothesis (Luo et al., 2005; Yuan et al., 2009). However, it remains to be explored whether the mrp is a sepal- or prophyll-like organ. Analyses of the mfo1 pal1 or mfo1 rep1 double mutants, and the expression patterns of MFO1, PAL1, and REP1 in related mutants, should shed further light on this question.

Evolutionary View of AGL6-Like and SEP-Like Genes Implied by Conserved C-Terminal Motifs

A previous report suggested that the C-terminal portion of MFO1/MADS6 contains a potential transcriptional activation domain (Moon et al., 1999). Also, the C termini portions of some AP1/FUL-like and SEP-like proteins were shown to have transcriptional activation activity (Cho et al., 1999; Honma and Goto, 2001; Ferrario et al., 2003). This activity of SEP-like proteins may be mediated by SEP I/II motifs, which primarily contain hydrophobic and polar residues and do not resemble any protein motifs with known function, although the effects of these motifs have not been proven at the molecular level (Ma et al., 1991; Huang et al., 1995; Immink et al., 2002; Ferrario et al., 2003; Shchennikova et al., 2004; Zahn et al., 2005). By contrast, the transcriptional activation activity of the AP1/FUL-like protein has been demonstrated to be localized to the C-terminal region of AP1, which does not possess motifs similar to SEP I or II, and transcriptional activation activity has not been demonstrated for FUL, which has a SEP II-like motif (Cho et al., 1999; Litt and Irish, 2003). Our results revealed that the C-terminally conserved motifs of AGL6-like proteins with high similarity to the SEP I/II motifs have potential transcriptional activation activity. Interestingly, not only these AGL6 motifs, but also the intervening region between these motifs exerted transcriptional activity in yeast. As mentioned (Figure 4C) the C-terminal amino acid sequences of MADS17, At AGL6, and At AGL13 have diverged from the consensus sequence to some extent. Nevertheless, the C terminus of MADS17 showed transcriptional activation at least in yeast, suggesting that a slight mutation might be acceptable for the activity of this domain.

Intriguingly, the intervening region is moderately conserved in AGL6-like proteins, but not in SEP-like proteins. Whereas AGL6-like genes have been identified in both angiosperms and gymnosperms, SEP-like genes have been identified only in angiosperms, suggesting that an ancestor of SEP-like genes existed in common ancestors of angiosperms and gymnosperms but was lost in extant gymnosperms (Becker and Theissen, 2003; Zahn et al., 2005). Therefore, SEP-like genes might have lost the semiconserved intervening sequences during evolution while still retaining transcriptional activation activity. Alternatively, an ancestor of extant AGL6-like genes might have evolved the intervening sequence. Further accumulation of genome information in both gymnosperms and angiosperms and molecular genetic evidence might unveil the evolutionary and developmental significance of the conservation and diversification in closely related AGL6-like and SEP-like gene families.

METHODS

Plant Materials

A recessive mutant, mfo1-1, was selected from an M2 population of rice (Oryza sativa ssp japonica cv Kinmaze) mutagenized with N-methyl-N-nitrosourea. The second allele, mfo1-2, was selected from the population of Tos17 retrotransposon insertion lines derived from cv Nipponbare, using the database of sequences flanking the Tos17 insertional positions to identify insertions in MFO1 (Miyao et al., 2003). For histological observations, RNAi experiments, and as a wild-type control for RT-PCR, we used cv Nipponbare. The lhs1-2 mutant (formerly called NF1019, a Tos17 insertion line, cv Nipponbare) was obtained from the Rice Genome Resource Center, National Institute of Agrobiological Sciences.

Histochemical Analyses

For paraffin sectioning, samples were fixed overnight at 4°C in FAA (formalin:glacial acetic acid:70% ethanol; 1:1:18) and dehydrated in a graded ethanol series. Following substitution with xylene, we embedded the samples in Paraplast Plus (McCormick Scientific) and sectioned them at 8-μm thickness using a rotary microtome. Sections were stained with 0.05% toluidine blue and observed with a light microscope as described previously (Kawakatsu et al., 2006). For scanning electron microscopy, fresh samples were applied directly to the scanning electron microscope (VE-8000; Keyence).

Mapping of MFO1

An F2 population between mfo1-1 (ssp japonica) and cv Kasalath (ssp indica) was used for the map-based cloning of MFO1. Using restriction fragment length polymorphism (C63223 and C60710) and cleaved-amplified polymorphic sequence markers (see Supplemental Table 1 online), MFO1 was mapped within an ~348 kb region on chromosome 2.

In Situ Hybridization

Digoxigenin-labeled antisense and sense probes were prepared with in vitro transcription from cDNAs of MFO1 and MADS17 using
gene-specific primers (see Supplemental Table 1 online). In situ hybridization and immunodetection of hybridization signals were performed as described (Kouchi and Hata, 1993) using hydroxylized (for MFO1) or nonhydroxylated (for MAD517) probes.

RNA Isolation and Analysis
We isolated total RNA from young panicle and floral organs using the RNeasy plant mini kit (Qiagen) according to the manufacturer’s manuals. RT-PCR analysis was executed using the total RNA and the Titan One Tube RT-PCR system (Roche Diagnostics). The rice Adenine Phosphoribosyltransferase1 (APT1) and ACTIN1 genes were used as controls. Quantitative RT-PCR analyses were performed with the single-color realtime detection system using Thermal Cycler Dice Real Time System Single TP850 system (Takara Bio) and SYBR Premix Ex Taq kit (Takara Bio). cDNA was synthesized from each total RNA samples using Super Script III reverse transcriptase (Invitrogen) with random hexamer primers. The results were analyzed by the crossing point method and normalized using the UBO expression level. Each run had replicates as indicated. The gene-specific primer pairs used in RT-PCR or quantitative RT-PCR are shown in Supplemental Table 1 online.

Transformation of Rice
Genomic DNA fragments and cDNAs with or without mutations were amplified using PCR with PfuUltra DNA polymerase (Stratagene) and verified by sequencing. For complementation, the DNA fragments were fused into pZH2B (donated by M. Kuroda). For RNA experiments, gene-specific DNA fragments of 370 bp (MFO1; nucleotides 624 to 993 of AK069103) or 361 bp (MAD517; nucleotides 634 to 994 of AY551918) were cloned in head-to-head orientation into pZH2B (donated by M. Kuroda) as described (Yoshida et al., 2007). The vectors were introduced into scutellum-derived calli of wild type, mfo1-1, mfo1-2, and lhs1-2 by Agrobacterium tumefaciens-mediated transformation under selection with hygromycin, as described elsewhere (Toki et al., 2006). The genetic backgrounds were cultivars Nipponbare (wild type, mfo1-2, and lhs1-2) and Kinnmaze (mfo1-1). Homozygous mfo1-1, mfo1-2, and lhs1-2 calli were selected using allele-specific PCR markers.

Transactivation Assay in Yeast
The wild-type or truncated version of 3’ sequences of MFO1 and MAD517, corresponding to the region spanning from the AGL6-I motif to the AGL6-II motif, were amplified using PfuUltra DNA polymerase (Stratagene), verified by sequencing, and translationally fused to the binding domain of the yeast GAL4 protein (GAL4-BD) on the pAS2-1 vector. All yeast transformations were performed as described previously (Yoshida et al., 2007), except for using MalV203 as the host strain. The ability to drive the expression of the yeast HIS3 reporter gene was tested by growing transformants on selective medium lacking Trp, Leu, and His in the presence of 30 mM of 3-aminitriazole. LacZ reporter gene activity in the yeast cells was monitored visually using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside filter assay as described (Yoshida et al., 2007).

Phylogenetic Analysis
Phylogenetic analysis of 32 MADS box protein sequences (see Supplemental Table 2 online) were conducted using MEGA version 3.1 (Kumar et al., 2004) with default settings. The gap opening penalty was 10, and the gap extension penalty was 0.1 for pairwise alignments, and they were 10 and 0.2, respectively, for multiple alignments. The Gonnet matrix was selected, and residue-specific and hydrophilic penalties were ON. The gap separation distance was 4, and end gap separation was OFF. Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap values (%) of 1000 replicates are shown at the branching points.

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL/DDBJ databases under the following accession numbers: FJ666318 and AK069103 (MFO1), FJ668596 and AY551918 (MAD517), AAG35652 (LHS1), AK073627 (APT1), AK100267 (ACTIN1), D12629 (UBQ), AP005775 (OSJNBb0005A04), and AP004255 (OJ1212A08). Locus identifications in the Rice Annotation Project Database are as follows: Os02g0682200 (MFO1/MAD517) and Os04g0680700 (MAD517). Tos17 insertion lines used in this study are as follows: NE4011 (mfo1-2) and NF1019 (lhs1-2).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Table 1. Primers Used in This Study.
Supplemental Table 2. Gene List Used in Phylogenetic Analysis.
Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Figure 4.

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**MOSAIC FLORAL ORGANS1, an AGL6-Like MADS Box Gene, Regulates Floral Organ Identity and Meristem Fate in Rice**

Shinnosuke Ohmori, Mayumi Kimizu, Maiko Sugita, Akio Miyao, Hirohiko Hirochika, Eiji Uchida, Yasuo Nagato and Hitoshi Yoshida

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