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Characterization of *Osmads6-5*, a null allele, reveals that *OsMADS6* is a critical regulator for early flower development in rice (*Oryza sativa* L.)

Yuanlin Duan · Zhuo Xing · Zhijuan Diao · Wenying Xu · Shengping Li · Xiaoqiu Du · Guangheng Wu · Chuanlei Wang · Tao Lan · Zheng Meng · Huaqing Liu · Feng Wang · Weiren Wu · Yongbiao Xue

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Abstract *AGL6*-clade genes are a subfamily of MADS-box genes and preferentially expressed in floral organs. *OsMADS6* and *OsMADS17* are two *AGL6*-like genes in rice. *OsMADS17* has been shown to play a minor role in floral development and appears to result from a duplication of *OsMADS6*. *OsMADS6* was initially named as *MFO1* for mosaic floral organs based on its moderate mutant phenotypes. So far, four moderate or weak mutant alleles of *OsMADS6* have been described, providing valuable insights into its role in flower development. Here, we report a null allele of *OsMADS6* (*Osmads6-5*), which exhibited a strong mutant phenotype in spikelet without affecting vegetative traits, causing all floral organs except lemma homeotically transformed into lemma-like organs (LLOs) as well as an indeterminate floral meristem, thus resulting in a mutant floret consisting of reiterating whorls of lemma and LLOs. In consistently, over-expression of *OsMADS6* led to additional lodicule-, stamen- and carpel-like organs. Expression analysis showed that *OsMADS6* controls the formation of the incipient primordia of lodicule, stamen and carpel via regulating the expression of class B, C and *SEP*-like MADS-box genes. Taken together, our results revealed that *OsMADS6* acts as a critical regulator for early flower development in rice and provide novel insights into the molecular mechanism of *OsMADS6*.

Keywords Rice · *Osmads6-5* · Null allele · Floral meristem determinacy · Floral organ identity

Abbreviations

Os	*Oryza sativa*
AGL	Agamous-like
MFO	Mosaic floral organ
SEP	Sepallata
FT	Flowering time

Yuanlin Duan and Zhuo Xing contributed equally to this work.

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Y. Duan · Z. Diao · S. Li · G. Wu · C. Wang · T. Lan · W. Wu
Key Laboratory of Ministry of Education for Genetics, Breeding and Multiple Utilization of Crops, Fujian Agricultural & Forestry University, Fuzhou 350002, China

Y. Duan · T. Lan · W. Wu (✉)
Fujian Provincial Key Laboratory of Marker-Assisted Breeding of Rice, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian, China
e-mail: wuwr@fjau.edu.cn

Z. Xing · W. Xu · Y. Xue (✉)
Laboratory of Molecular and Developmental Biology, Institute of Genetics & Developmental Biology, Chinese Academy of Sciences and National Center for Plant Gene Research, Beijing 100101, China
e-mail: ybxue@genetics.ac.cn

Z. Xing
Graduate University of Chinese Academy of Sciences, Beijing 100049, China

X. Du · Z. Meng
Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

H. Liu · F. Wang
Academy of Agricultural Science, Fuzhou 350000, Fujian Province, China

SOC1	Suppressor of overexpression of CO 1
LFY	Leafy
AP1	Apetala 1
AG	Agamous
BDE	Bearded-ear
FBP	Floral binding protein2
DL	Drooping leaf
SEM	Scanning electron microscopy
RT-PCR	Reverse transcription polymerase chain reaction
qRT-PCR	Real-time quantitative RT-PCR
SSR	Simple sequence repeat
GFP	Green fluorescent protein
LLO	Lemma-like organ
PLO	Palea-like organ

Introduction

Recent studies have shown that *SEP* genes have divergent functions and play fundamental roles in determining floral fate. In *Arabidopsis*, four *SEP* genes (*SEP1/2/3/4*) are necessary for the identity specification of all four floral whorls and for the determinacy of floral meristem (Pelaz et al. 2000; Ditta et al. 2004). In rice, five *SEP*-like genes have been identified. Among them, *OsMADS1* is mainly involved in the specification of lemma/palea and the regulation of floral meristem determinacy (Jeon et al. 2000a; Prasad et al. 2001; Malcomber and Kellogg 2004; Agrawal et al. 2005; Prasad et al. 2005; Chen et al. 2006) and the other four *SEP*-like genes might function redundantly in specifying floral organ identity or spikelet development (Agrawal et al. 2005; Cui et al. 2010; Kobayashi et al. 2010; Gao et al. 2010).

AGL6-like genes are a subfamily of MADS-box genes. Phylogenetic analysis suggests that *AGL6*-clade is the closest relative of *AGL2*-clade (*SEP*-like) and has been hypothesized to be sister to *AGL2*-clade (Zahn et al. 2005). In grasses, the expression of *AGL6*-clade genes was detected in ovules, carpels, lodicules (equivalent to second whorl floral organs), paleas (putative first whorl floral organs), and floral meristems, suggesting that they may act in both perianth and gynoecium development (Reinheimer and Kellogg 2009). In addition, some studies suggested that over-expression of *AGL6*-clade genes can promote the expression of flowering time genes (*FT* and *SOC1*), flower meristem identity genes (*LFY* and *AP1*) and floral organ identity genes (*AG* and *SEP1*) and consequently result in early flowering and dwarf transgenic plants and homeotic transformation of floral organs (Jeon et al. 2000b; Hsu et al. 2003; Carlsbecker et al. 2004; Tian et al. 2005; Fan et al. 2007). Together, these results show that *AGL6*-like genes might play roles in the initiation and formation of flowers, though they are not included in the typical ABCDE model of flower development. However, actual functions of *AGL6*-clade genes largely remain enigmatic due to lack of their loss-of-function mutants. Recently, identification of loss-of-function mutants in Petunia and maize shed lights on the functions of *AGL6*-clade genes (Rijpkema et al. 2009; Thompson et al. 2009). The *Petunia hybrida AGL6*-clade gene, *PhAGL6*, has been found to function redundantly with the *SEP*-like genes *FBP2* (FLORAL BINDING PROTEIN2) and *FBP5* in petal, anther and carpel development. In addition, PhAGL6 and FBP2 proteins may share similar biochemical characteristics because they interacted with the same partners (Rijpkema et al. 2009). In maize, an *AGL6*-clade gene *bearded-ear* (*bde*) was considered to resemble E-function genes because it affects floral organ development in all four whorls and floral meristem identity, and likely functions in multiple complexes including BDE proteins and the C-class protein ZAG1 (Thompson et al. 2009).

OsMADS6 and *OsMADS17* are two rice *AGL6*-clade genes. *OsMADS17* has been shown to play a minor role in rice floral development and appears to result from a duplication of *OsMADS6* (Ohmori et al. 2009; our unpublished results). Recently, two mutant alleles of *OsMADS6* named *mfo1-1* and *mfo1-2,* showing a phenotype of mosaic organs in flowers, have been isolated (Ohmori et al. 2009). Also, Li et al. (2010) described another mutant allele *Osmads6-1*, similar to *mfo1-1*. Both studies suggested that *OsMADS6* appears to regulate floral development redundantly with *OsMADS1* (Ohmori et al. 2009; Li et al. 2010). Whereas Zhang et al. (2010) reported that *OsMADS6* shows an essential role in endosperm nutrient accumulation and is subject to epigenetic regulation from two additional mutant alleles of *OsMADS6* (here we renamed one as *Osmads6-2* and the other is identical to *mfo1-2*). Moreover, Li et al. (2011) suggested that *OsMADS6* genetically interacts with rice floral homeotic genes *OsMADS16/3/58/13* and DROOPING LEAF (*DL*) in specifying floral organ identities and meristem fate and, in particular, OsMADS6 protein can directly bind to OsMADS58. However, these four *OsMADS6* mutant alleles showed moderate (*mfo1-1*, *Osmads6-1* and *Osmads6-2*) or weak (*mfo1-2*) floral phenotypes. Hence, the loss of function phenotype of *OsMADS6* remains to be further defined.

In this work, we identified the fifth mutant allele, a null allele, of *OsMADS6* (*Osmads6-5*), which clearly revealed the function of *OsMADS6* in flower development, showing that it is critical for determining the floral developmental fate and meristem determinacy by regulating the expression of floral organ identity genes at early stages of flower development and providing a new insight into the molecular mechanism of flower development in rice.

Materials and methods

Plant materials

The *Osmads6-5* was discovered in a doubled haploid (DH) line derived from the F_1 of a cross between an *indica* variety Gui-630 and a *japonica* variety Taiwanjing by anther culture and maintained through heterozygotes.

Microscopic observation

Young panicles (1–10 mm) were observed by scanning electron microscopy (SEM, Duan et al. 2010). Panicles were fixed in 2.5 % glutaric dialdehyde and washed with a sodium phosphate buffer (0.1 M, pH 7.0); further fixed in 1 % osmic acid for 1–2 h and again washed with the sodium phosphate buffer (0.1 M, pH 7.0); dehydrated with an ethanol series, incubated in ethanol-isoamyl acetate and then in isoamyl acetate; dried, mounted and coated with gold; and finally observed with a XL30 ESEM scanning electronic microscope (PHLIPS Company, Amsterdam, The Netherlands).

Histochemical analysis

Rice flowers fixed in 2.5 % glutaraldehyde solution were dehydrated using a graded ethanol series and embedded in Leica 7022 historesin (Leica, Nussloch, Germany). Samples were sectioned to 4 μm, stained with 0.1 % Toluidine Blue-O (Sigma, St. Louis, MO, USA) and observed under an Olympus AX-80 light microscope.

Isolation of *Osmads6-5*

Heterozygotes of *Osmads6-5* were crossed with an *indica* cultivar Minghui-77, and 1,128 F_2 plants with the *Osmads6-5* phenotype were selected for gene mapping. Publicly available RM-series simple sequence repeat (SSR) markers as well as some new SSR markers (data not shown) developed by us were used for fine mapping. Reverse transcription PCR (RT-PCR) analysis of the expression of *Osmads6-5* allele was performed using a pair of primers (5′-ATGACTCCTACCCACCATTG-3′ and 5′-TCAAAGAACCCATCCCAGCATGAAG-3′). For complementation test, a 13,488-bp *OsMADS6* genomic DNA fragment covering 3,702-bp promoter and 2,198-bp 3′ region was sub-cloned into a binary vector *pCAMBIA1300* and introduced into *Osmads6-5* mutant embryonic calli by *Agrobacterium tumefaciens*-mediated transformation (Hiei et al. 1994).

Function analysis of a truncated polypeptides of *Osmads6-5*

The 477 bp truncated *OsMADS6* coding sequence was cloned into a binary vector *pTCK303* driven by a rice ubiquitin promoter (Wang et al. 2004). The binary construct was introduced into EHA105 and further into rice. Seven independent transgenic plants were acquired.

Over-expression of *OsMADS6*

Full-length O*sMADS6* cDNA was introduced into a modified binary vector *pCAMBIA1301* with restriction sites of *Bam*HI and *Sal*I (Chen et al. 2006). The binary construct *35S*:*OsMADS6* was introduced into EHA105 and further into rice. Eight independent *35S*:*OsMADS6* plants were obtained.

Real-time quantitative PCR (qRT-PCR) analysis

Reverse transcription of total RNA was performed using SuperScript III First-Strand Synthesis kit (Invitrogen, USA). As previously described (Lan et al. 2004), the cDNA samples were diluted into 8 ng/μl and 2 ng/μl. Triplicate quantitative assays were performed using the SYBR Green Master Mix (Applied Biosystems, CA, USA) with an ABI 7900 sequence detection system. The relative quantification method (ΔΔCT) was used to evaluate quantitative variation between replicates examined. Amplification of 18S rRNA was used as an internal control to normalize all data. Primers used for qRT-PCR analysis are listed in Table S1.

mRNA in situ hybridization

mRNA in situ hybridization was essentially performed as previously described by Lai et al. (2002). Wild-type and *Osmads6-5* young panicles (2–10 mm) were fixed with formalin-acetic acid-alcohol solution and embedded in paraffin. Probe of *OsMADS1* was designed as previously described by Li et al. (2009); *OsMADS3/58* by Yamaguchi et al. (2006); *OsMADS7/8* by Cui et al. (2010) and *OsMADS17* by Ohmori et al. (2009). The *OsMADS4/16* probe primers were 5′-CGGCTACCACCACGACGACA-3′/5′-CTGAGTGCTAATGCTGGGAG-3′ and 5′-ACCGT ATCATCGCTCGATCT-3′/5′-GCACACCACGCATACA TAAT-3′, respectively.

Protoplast transient expression assay

The full-length and truncated *OsMADS6* coding sequences (deletion the MADS-domain) were cloned into a rebuilt vector *pBI221* driven by CaMV *35S* promoter to generate *p221-35S:OsMADS6-GFP,* respectively. The *GFP* fusion construct was transformed into Arabidopsis protoplasts using the protocol described previously (Yoo et al., 2007). Localization of OsMADS6-GFP fusion protein was observed with a Zeiss LSM 510 META confocal microscope (Zeiss, Jena, GER).

Results

The *Osmads6-5* floret comprises many whorls of lemma-like organs

A mature wild-type floret of rice comprises of a lemma and a palea, a pair of lodicules, six stamens and a carpel (Fig. 1a). We identified a floral organ mutant of rice initially named *lemmata* because its flower only consisted of reiterating lemma or lemma-like organs (LLOs). We showed that the mutant was caused by a mutation of *OsMADS6* (see below). Since four different *OsMADS6* alleles have been described previously (Ohmori et al. 2009; Li et al. 2010; Zhang et al. 2010), to follow the previous work, we hereafter renamed *lemmata* as *Osmads6-5*. *Osmads6-5* appeared normal for heading time and all vegetative morphological characters except for floret phenotype (Fig. 1b). The *Osmads6-5* floret appeared noticeably bulgy in comparison with the wild-type floret (Fig. 1c, d). The lemma of the mutant floret was normal in size, but the palea was significantly enlarged (Fig. 1e). It is known that lemma and palea normally have five and three vascular bundles, respectively (Fig. 1g). The number of vascular bundles remained normal in the *Osmads6-5* lemma, but greatly increased (mostly up to 8–10) in its palea (Fig. 1h). In addition, the *Osmads6-5* palea became curled inward on the margin like a lemma, rather than formed the membranous margin and the barbs that exist in the normal palea (Fig. 1g, h). These results suggest that the identity of lemma remains unchanged, whereas the identity of palea is evidently altered, losing the feature of palea but gaining the feature of lemma in the *Osmads6-5* floret.

Inside the *Osmads6-5* floret, the lodicules, stamens and carpel all disappeared and the space was filled with many green organs (Fig. 1d). Anatomical observation clearly revealed that these green organs all possessed the inner structure similar to that of lemma/palea, namely, consisting of four layers of different tissues (cell types), including upper epidermis (silicified cells), fibrous sclerenchyma cell layer, spongy parenchymatous cell layer, and lower epidermis non-silicified cells (Prasad et al. 2005); in addition, they all had the morphological feature of curled margin, like a lemma (Fig. 1h). Hence, it is reasonable to identify these green

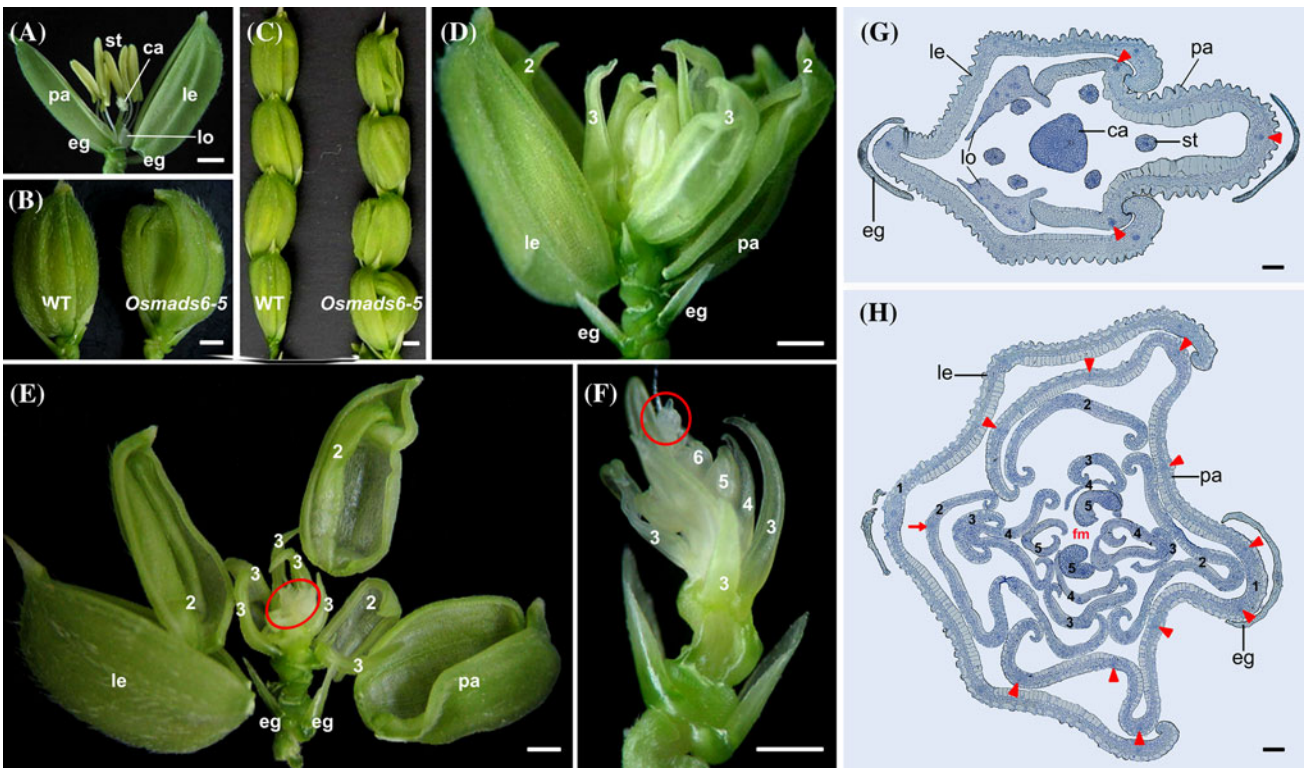


Fig. 1 Morphological and anatomical observation of wild-type and *Osmads6-5* spikelets. **a** An opened wild-type spikelet. **b**, **c** Intact spikelets and panicles of wild-type and *Osmads6-5*. **d**, **e** Opened mature *Osmads6-5* spikelets. **f** Opened young *Osmads6-5* spikelets. Lemma, palea and two LLOs transformed from two lodicules were removed. **g**, **h** Transverse section of a wild-type spikelet (**g**) and an *Osmads6-5* mutant spikelet (**h**). *Arrowheads* indicate vascular bundles in palea. *Arrow* indicates vascular bundle in LLO. *Red circle* in (**e**) and (**f**) indicates a developing centre of a young mutant spikelet, where many whorls of LLO would be continuously formed. *Numbers 2* and *3* in (**d**–**f**) and (**h**) indicate LLOs transformed from lodicules and stamens in whorl 2 and 3, respectively; *numbers 4–6* indicate whorls of LLOs transformed from pistil. *eg* empty glume, *le* lemma, *pa* palea, *lo* lodicule, *st* stamen, *ca* carpel. *Scale bars* 1 mm in (**a**–**f**), and 100 μm in (**g**, **h**)

Table 1 Number of LLOs in each whorl or inner whorls inside the mature *Osmads6-5* floret

Whorl 2	Whorl 3	Whorl 4	Inner whorls
1 (9.8 %)	3 (0.9 %)	3 (2.8 %)	5 (0.8 %)
2 (80.6 %)	4 (13.6 %)	4 (21.3 %)	6 (9.6 %)
3 (6.5 %)	5 (24.2 %)	5 (34.7 %)	7 (19.3 %)
4 (3.1 %)	6 (61.3 %)	6 (41.2 %)	8 (32.7 %)
			9 (25.5 %)
			10 (8.7 %)
			>10 (3.4 %)

The number in each parenthesis indicates the frequency in 347 *Osmads6-5* florets

organs as LLOs. The LLOs were arranged in many (usually 6–10, occasionally 12–15) whorls (Fig. 1f, Table 1). The numbers of LLOs in whorls 2 and 3 were different. In the most *Osmads6-5* florets, there were two and six LLOs in whorls 2 and 3, respectively (Fig. 1e, Table 1), suggesting that the LLOs in whorls 2 and 3 might be homeotically transformed from the two lodicules and six stamens, respectively. Nevertheless, a few *Osmads6-5* florets with fewer and one or two larger LLOs in whorls 2 and 3 were observed, suggesting that two or more LLOs might have merged into a larger one. Occasionally, we also observed that 3–4 LLOs developed in whorl 2 (Fig. 1e, h). Interestingly, the carpel in the *Osmads6-5* floret was replaced by many (often 3–7) whorls of LLOs (Fig. 1f, h, Table 1), suggesting that the determinacy of the floral meristem was lost.

To further clarify the morphogenesis of *Osmads6-5* flowers, we examined young spikelets of both the mutant and wild type at different developmental stages by SEM (Fig. 2). The results clearly demonstrated that the floral primordium in the wild-type spikelet developed in an order of that empty glumes developed first (Fig. 2a), followed by a lemma and a palea (Fig. 2a), two lodicules, six stamens (Fig. 2b) and a carpel (Fig. 2c, d). In the mutant, the development of floral primordium also occurred from the periphery to the center. The primordia of empty glumes and lemma could normally emerge and develop into the correct organs in the *Osmads6-5* spikelet (Fig. 2e). The palea primordium could also emerge normally in the *Osmads6-5* spikelet, but an obvious notch and multiple bumps formed as the development progressed (Fig. 2e–g), while in the wild-type spickelet only a single bump developed in the central region of palea (Fig. 2b, c). This could explain why the *Osmads6-5* palea was larger in size and had more vascular bundles. The primordia at whorls 2 and 3 could be formed as expected in the *Osmads6-5* spikelet, but they were all homeotically transformed and developed into LLOs, instead of lodicules and stamens as in the wild type (Fig. 2h). Occasionally, some anther-shaped primordia that would develop into LLOs were observed in whorl 3 in the *Osmads6-5* spikelet (Fig. 2i). This provides a piece of direct evidence for a homeotic transformation of stamens taking place in the mutant. The central region (whorl 4) of floral primordium did not develop into a single LLO corresponding to carpel in the *Osmads6-5* spikelet. Instead, a process similar to that occurred in whorl 3 was reiterated in the central region, resulting in multiple (ranging 3–7) whorls of LLOs (Figs. 1h, 2j, k, Table 1).

In summary, all the floral organs of *Osmads6-5* that develop after lemma are transformed into LLOs, and potentially endless whorls of LLOs are generated due to the loss of floral meristem determinacy, indicating that the *OsMADS6* gene is critical for the identity specification of palea, lodicule, stamen and carpel, and for the determinacy of floral meristem.

The phenotype of *Osmads6-5* is caused by a large insertion-deletion mutation in *OsMADS6*

Genetic analysis showed that *Osmads6-5* is controlled by a single recessive gene and the mutant phenotype was stable across different cropping seasons (Table 2). To reveal the molecular cause of the mutant phenotype, we employed the positional cloning strategy to isolate *Osmads6-5* gene. Using publicly available RM-series SSR markers as well as some new SSR markers developed in this study, we fine mapped *Osmads6-5* to a 66.1-kilobase (kb) region on the long arm of chromosome 2. According to the annotation provided by the NCBI database, this region contains 12 genes including *OsMADS6* (data not shown). Using a pair of primers designed according to the DNA sequence of *OsMADS6*, we found that the *OsMADS6* locus was completely co-segregated with the mutant trait (data not shown). Sequence analysis of the two bands amplified from the wild-type and the mutant genomic DNA revealed a 2,915-bp deletion (including 2,783-bp of the promoter and 132-bp of the first exon) replaced by an 852-bp insertion (Dataset S1) in the *OsMADS6* locus (Fig. 3a). We compared the sequences of other 11 genes in this region between the wild type and the mutant, but no variation was found. Therefore, *OsMADS6* should be the only candidate gene. To validate the candidate gene, a complementation vector containing a genomic fragment covering a 3,702-bp promoter region and a 2,198-bp 3′ end region of *OsMADS6* was introduced into *Osmads6-5*, and the mutant phenotype was rescued (Fig. 3b), confirming that the mutant phenotype was indeed caused by the mutation of *OsMADS6* gene.

Osmads6-5 is a null allele of *OsMADS6*

In order to confirm that *Osmads6-5* is a null allele of *OsMADS6*, we examined the expression of *Osmads6-5*.

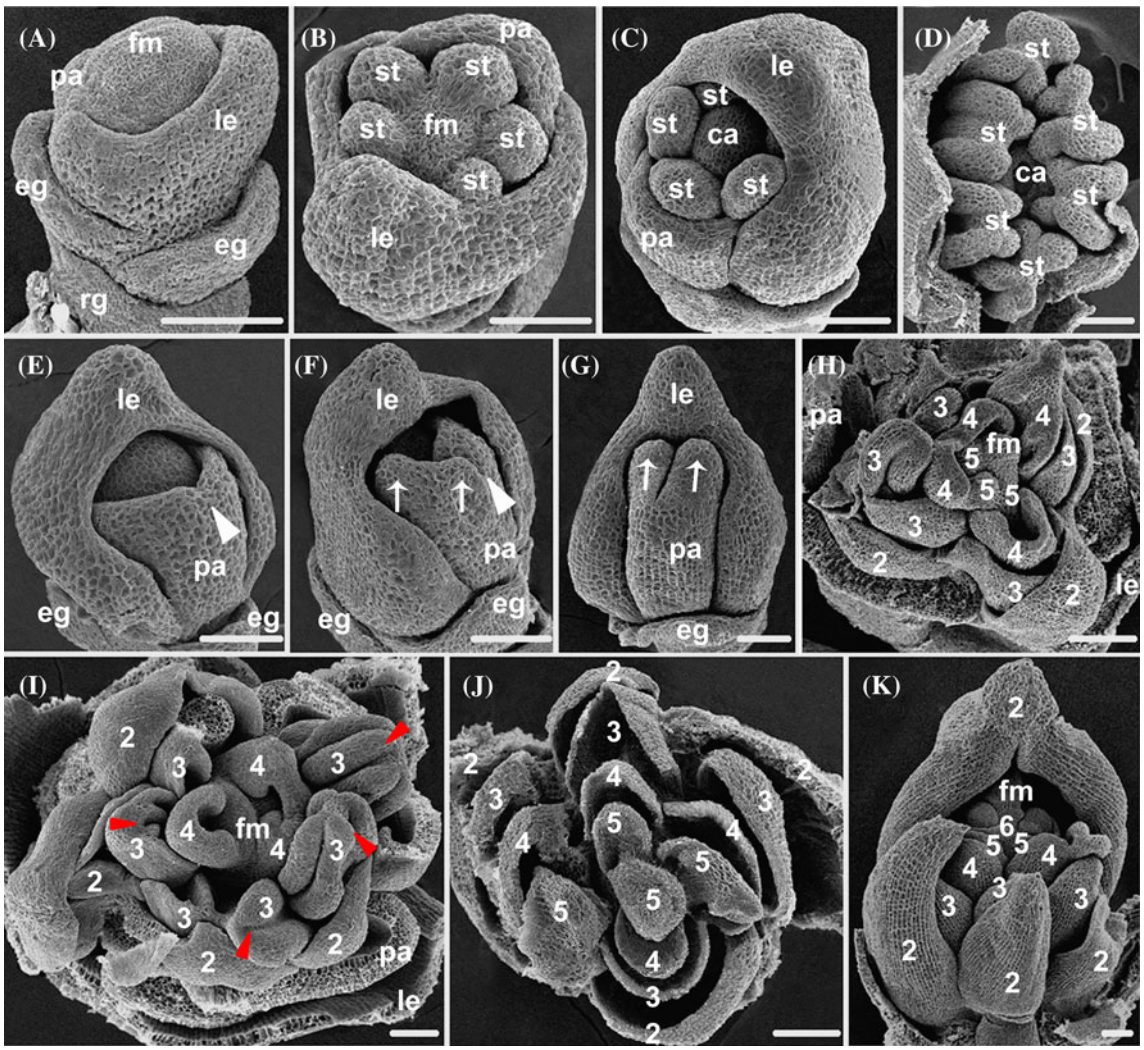


Fig. 2 SEM observations of wild-type and *Osmads6-5* spikelets during early developmental stages. **a–d** Wild-type spikelets. Lemma and palea were removed in (**d**). **e–k** *Osmads6-5* spikelets. *White arrowheads* in (**e**) and (**f**) indicate notch formed in the mutant palea. *White arrows* in (**f**) and (**g**) indicate new bump emerged at the mutant palea. *Red arrowheads* in (**i**) indicate anther-shaped LLO organs in the whorl 3. Lemma and palea were removed in (**h–k**). *Numbers 2* and *3* indicate LLOs transformed from lodicules and stamens in whorls 2 and 3, respectively; *numbers 4–6* incidate whorls of LLOs transformed from pistil. *rg* rudimentary glume, *eg* empty glume, *le* lemma, *pa* palea, *lo* lodicule, *st* stamen, *ca* capel, *fm* floral meristem. *Scale bars* 50 μm

Table 2 Phenotypic segregation in the selfed progeny of *Osmads6-5* heterozygote in different cropping seasons

Cropping season	No. of plants			χ^2 (3:1)[a]
	Total	Normal	*Osmads6-5*	
Early (March–July, 2000)	431	311	120	1.86
Middle (May–September, 2001)	406	301	105	0.16
Late (July–November, 2000)	525	412	113	2.59
Total	1,362	1,024	338	0.03

[a] $\chi^2_{0.05,1} = 3.84$

The results of RT-PCR analysis indicated that a chimeric RNA transcript consisting of a 41 bp of the inserted sequence and its downstream sequence of *OsMADS6* was detected (Fig. 3c). Sequence analysis revealed that the predicted three reading frames all have premature stop codons, indicating that there is no in-frame start codon ATG in the insertion sequence (Fig. 3c). Next, we checked the downstream sequences of the chimeric transcript and found four possible ORFs encoding truncated OsMADS6 polypeptides (Fig. 3d). The largest predicted ORF, with

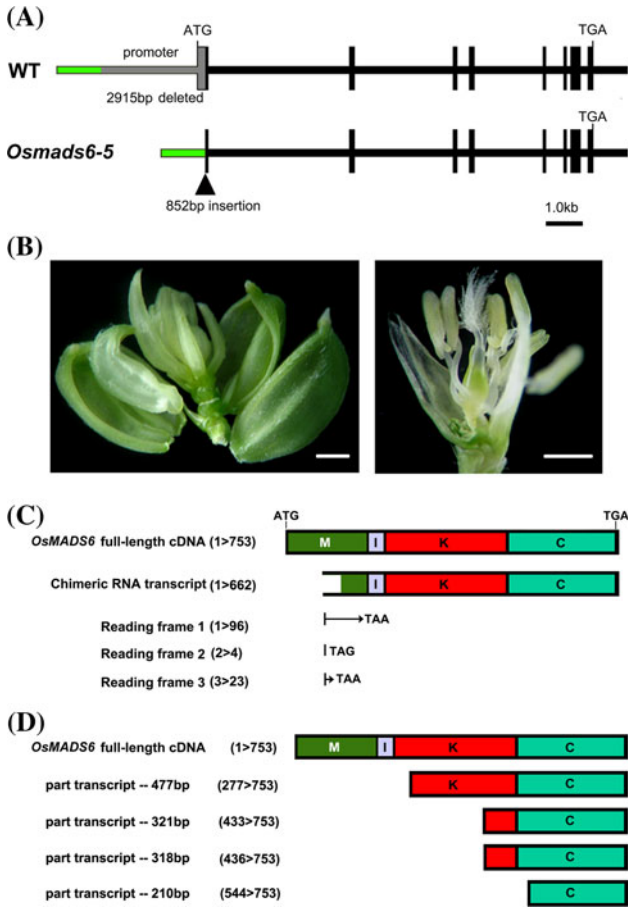


Fig. 3 Map-based cloning of *OsMADS6* and analysis of the schematic transcript of *Osmads6-5*. **a** Genomic organization of *OsMADS6* gene and *Osmads6-5* allele. *OsMADS6* consists of eight exons indicated by *vertical bars* (the 5′- and 3′-UTR are not shown). *Osmads6-5* has a large insertion-and-deletion mutation in *OsMADS6*. The deleted segment is indicated in *gray*. The intergenic region upstream of the deletion is indicated in *green*. **b** Complementation of *Osmads6-5* by *pCAMBIA1300-OsMADS6*. *Left* an opened mutant spikelet. *Right* a spikelet of complemented line (rescued line-1) with the palea/lemma removed from the flower. *Scale bars* 1 mm. **c** Schematic transcript structures of *OsMADS6* in wild-type and *Osmads6-5*. The chimeric RNA transcript in *Osmads6-5* encompasses a 41 bp inserted sequence (indicated in *white zone*) at 5′ end of the *OsMADS6* transcript. DNA sequence analysis revealed that all the three possible reading frames of the chimeric transcript had premature stop codons. **d** Possible ORF of schematic transcript of *Osmads6-5*. Four possible reading frames encoding truncated OsMADS6 polypeptides were found in the downstream sequences of the chimeric transcript of *Osmads6-5*. The MADS-box domain, I-region, K-region and C-terminal region are shown in *green*, *gray*, *red* and *blue* in **c** and **d**, respectively

the deletion of the MADS domain, I-region and partial K-region, was predicted to be capable of encoding of 159 amino acids identical to the region of OsMADS6 from 93 aa to 251 aa (Fig. 3d). In order to examine whether the truncated *OsMADS6* could possibly cause a weak dominant negative effect resulting in the observed phenotype in the double recessive plant but not in the heterozygous one, we expressed the 477 bases of transcription sequence under the control of an ubiquitin promoter. Compared with the wild type, all the transgenic lines displayed a normal floral phenotype except for a slight enclosure of panicles (Fig. S1). This result showed that the predicted truncated polypeptide of *Osmads6-5*, if any, could not cause a weak dominate negative effect on rice floral organ development. Therefore, the recessive mutant *Osmads6-5* is a null allele of *OsMADS6*.

Over-expression of *OsMADS6* results in over production of palea, lodicule, stamen and carpel but has little influence on vegetative traits

To further characterize the function of *OsMADS6*, we produced transgenic rice plants with over-expressed *OsMADS6* driven by the constitutive CaMV 35S promoter. Eight transgenic plants were obtained, in which the expression of *OsMADS6* was significantly increased (Fig. S2). No obvious phenotypic changes were observed in the transgenic plants except for spikelets. A few transgenic spikelets were found to possess an additional incomplete floret (Fig. 4b–d, Table 2). In most of the transgenic spikelets, the lodicule, stamen and carpel were all over-developed with some morphological abnormalities (Fig. 4e; Table 3). Many additional lodicules developed in the second whorl (Fig. 4f, g). Those lodicules looked normal except a little flatter in shape (Fig. 4e–g). Interestingly, two palea-like organs (PLOs) also developed in this whorl (Fig. 4e–g). Judging from their position, we speculated that the two PLOs might be homeotically transformed from the two original lodicules. In the third and fourth whorls, the most of the transgenic florets had 7–10 stamens and 2–4 carpels (Fig. 4f). Occasionally, two or more carpels merged at the base and developed into a larger one with some unclear tissues on the top (Fig. 4g). The lemma was normal in size, but the palea became obviously similar to lemma in both morph and size in the transgenic florets (Fig. 4e). In sum, these results indicated that *OsMADS6* plays a specific role in the regulation of floral organ identity except for the lemma and of flower meristem determinacy in rice.

Several class B, C and E genes are down-regulated but *OsMADS1* and *OsMADS17* are up-regulated in *Osmads6-5*

To examine the relationships between *OsMADS6* and other MADS-box genes in rice, we investigated the transcription levels of three *AP1*-like (*OsMADS14/15/18*), three class B (*OsMADS2/4/16*), two class C (*OsMADS3/58*), five class *SEP*-like (*OsMADS1/5/7/8/19*) and one *AGL6*-clade (*OsMADS17*) genes in the young panicles of *Osmads6-5*

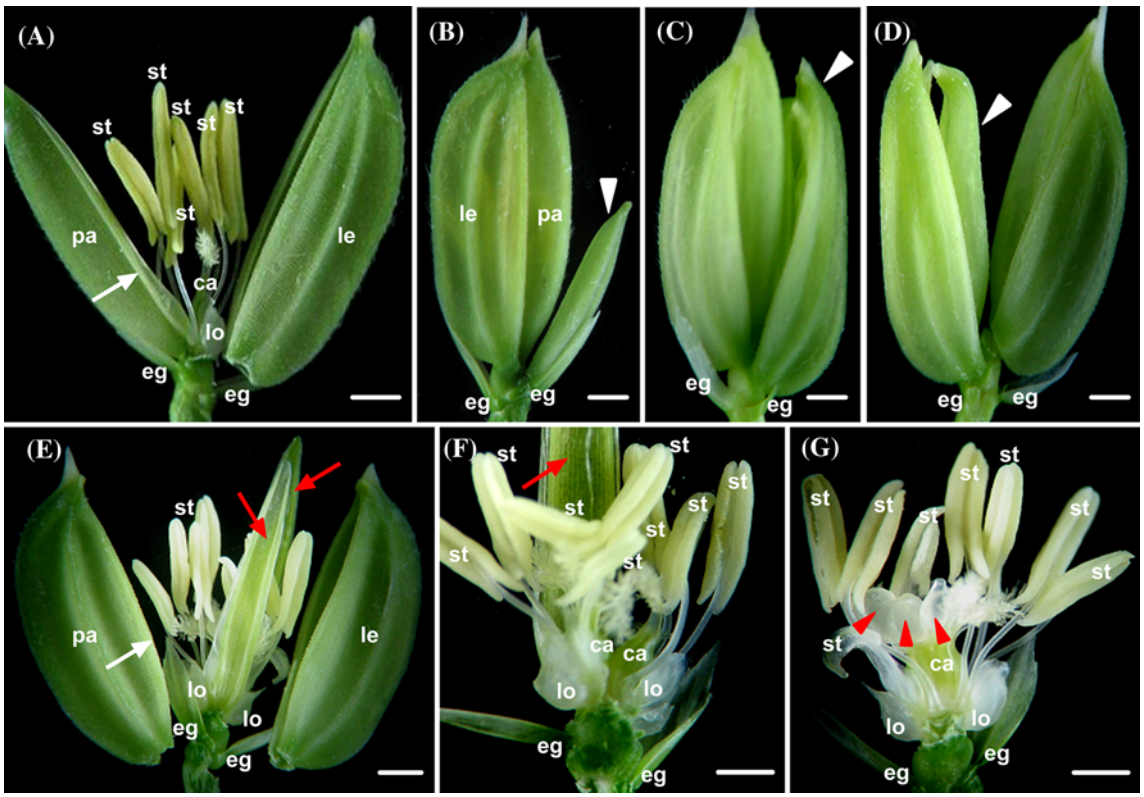


Fig. 4 Phenotypes of wild-type and the *35S:OsMADS6* spikelets. **a** Wild-type spikelet. *White arrow* indicates the palea marginal tissue. **b–d** *35S:OsMADS6* spikelets with an additional incomplete floret indicated by *white arrowheads*. **e–g** Opened *35S:OsMADS6* spikelets. Lemma and palea were removed in (**f**) and (**g**), one and two PLOs were removed in (**f**) and (**g**), respectively. *White arrow* in (**e**) indicates the loss of palea marginal tissue in palea (likely LLO). *Red arrows* in (**e**) and (**f**) indicate PLOs transformed from lodicules. *Red arrowheads* in (**g**) indicate unclear tissues. *eg* empty glume, *le* lemma, *pa* palea, *lo* lodicule, *st* stamen, ca capel. *Scale bars* 1 mm

Table 3 Numbers of floral organs in each whorl and additional florets in the *OsMADS6*-Overexpressed spikelets

The number in each parenthesis indicates the frequency in 267 *OsMADS6*-Overexpressed spikelets

	Whorl 2		Whorl 3 stamen	Whorl 4 pistil	Additional florets
	Lodicules	PLOs			
OM6-OX	1 (3.2 %)	0 (3.4 %)	6 (3.9 %)	1 (7.8 %)	0 (91.7 % %)
	2 (8.5 %)	1 (1.7 %)	7 (18.5 %)	2 (44.3 %)	1 (8.3 %)
	3 (19.6 %)	2 (94.9 %)	8 (29.2 %)	3 (21.3 %)	
	4 (34.9 %)		9 (16.6 %)	4 (14.9 %)	
	5 (27.5 %)		10 (9.2 %)	>4 (1.7 %)	
	>5 (6.3 %)		>8 (7.1 %)		

and wild type at the early stages of flower development using qRT-PCR. The results showed that in *Osmads6-5* mutant, one *SEP*-like gene (*OsMADS1*) and the *AGL6*-like member (*OsMADS17*) were found to be significantly up-regulated, but several class B (*OsMADS4/16*), class C/D (*OsMADS3/13/58*) and class E (*OsMADS7/8*) genes were found to be significantly down-regulated; whereas the mutation of *OsMADS6* appeared not to affect the expression of *AP1*-like genes (Fig. 5). These results suggested that Os*MADS6* regulates the morphogenesis and development of floral organs probably by regulating the expression of other MADS-box genes, especially the floral organ identity genes.

The expression patterns of floral organ identity genes are significantly altered in *Osmads6-5*

To further discern the relationships between *OsMADS6* and other floral organ identity genes in rice, we examined the expression of six down-regulated genes (*OsMADS3/4/7/8/16/58*) and two up-regulated genes (*OsMADS1/17*) by RNA in situ hybridization in the wild type and *Osmads6-5*.

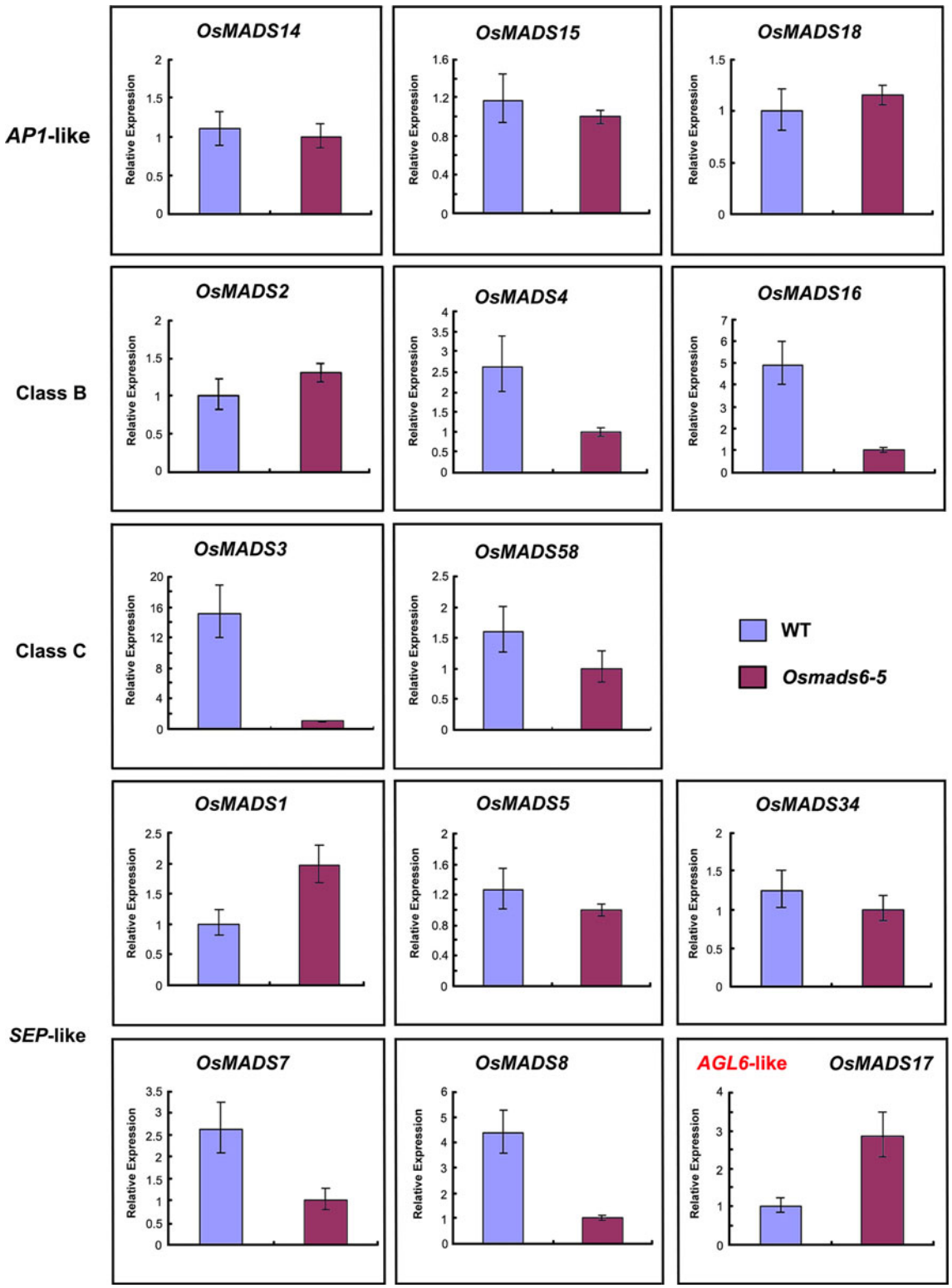


Fig. 5 qRT-PCR analysis of expression levels of *AP1*-like, Classes B/C, *SEP*-like and *AGL6*-like genes in young panicles of wild-type and *Osmads6-5*. Amplification of 18S rRNA (Table S1) was used as an internal control to normalize all data. The error bars showed the standard errors (SEs) for three replicates

The two class B genes (*OsMADS4/16*) had similar expression patterns. In wild-type spikelets, the two genes first expressed in the incipient primordia of lodicule and stamen, and their expression persisted during the development of the two floral organs (Fig. 6A[a–c], B[a–c]; Nagasawa et al. 2003). In *Osmads6-5* spikelets, however, the expression patterns of *OsMADS4* and *16* were obviously altered. The two genes showed a delayed expression, and almost no expression signal was detected in the presumptive initiating regions of whorls 2 and 3 where two and six LLOs would develop, respectively (Fig. 6A[d], B[d]). Weak expression signal was observed in the LLOs primordia of inner whorls until later developmental stages (Fig. 6A[e, f], 6B[e, f]).

The two class C genes (*OsMADS3/58*) started to be expressed when the lemma and palea primordia just appeared in wild-type flowers (Fig. 6C[a], D[a]). *OsMADS3* RNA accumulated in the stamen primordia at early stages and in the carpel primordia later (Fig. 6C[b, c]; Yamaguchi et al. 2006). *OsMADS58* signal was detected in the primordia of stamen and carpel throughout the developing stages (Fig. 6D[b, c]; Yamaguchi et al. 2006). In *Osmads6-5* spikelets, the expression of the two class C genes was all delayed, similar to that of the two class B genes. There was little detectable signal until whorl 3 was initiated (Fig. 6C[d], D[d]). Later, only weak expression was detectable, restricted to the inner whorls and the central floral meristem where many whorls of LLOs would develop subsequently (Fig. 6C[e, f], D[e, f]).

The expression domains of two *SEP*-like genes *OsMADS7/8* in the wild type were largely overlapped during spikelet development (Fig. 6E[a–c], F[a–c]); Cui et al. 2010). When the lemma and palea primordia were just initiating, both of the genes showed a strong expression in the floral meristem where the floral organ primordia of three inner whorls would subsequently initiate (Fig. 6E[a], F[a]). After that, strong transcription was restricted to lodicules, stamens and carpels (Fig. 6E[b, c], 6F[b, c]; Cui et al. 2010). In *Osmads6-5*, after the lemma and palea primordia emerged, the floral meristem did not show any detectable signal (Fig. 6E[d], 6F[d]). During later stages, the expression domain was confined to the LLOs primordia of inner whorls (Fig. 6E[e, f], F[e, f]). In addition, the expression levels of *OsMADS7/8* were significantly reduced in *Osmads6-5* spikelets.

Another *SEP*-like gene, *OsMADS1*, was principally expressed in palea and lemma in the wild type when their incipient primordia emerged, with a weak expression in carpel (Fig. 6G[a–c]; Prasad et al. 2005). In *Osmads6-5*, the initial expression of *OsMADS1* was not altered (Fig. 6G[d]). But the expression domain was clearly extended to the primordia of the inner-whorl LLOs during their development (Fig. 6G[e, f]), resulting in the up-regulated expression of *OsMADS1*.

Fig. 6 Expression of Class B, C, *SEP*-like genes and *OsMADS17* in wild type and *Osmads6-5* spikelets. **A**–**H** Expression of *OsMADS4*, *OsMADS16*, *OsMADS3*, *OsMADS58*, *OsMADS7*, *OsMADS8*, *OsMADS1* and *OsMADS17* in wild-type (**a**–**c**) and *Osmads6-5* spikelets (**d**–**f**), respectively. *Black arrowheads* indicate LLOs. *le* lemma, *pa* palea, *lo* lodicule, *st* stamen, *ca* carpel, *fm* floral meristem, *LLO* lemma-like organ. *Bars* 100 μm

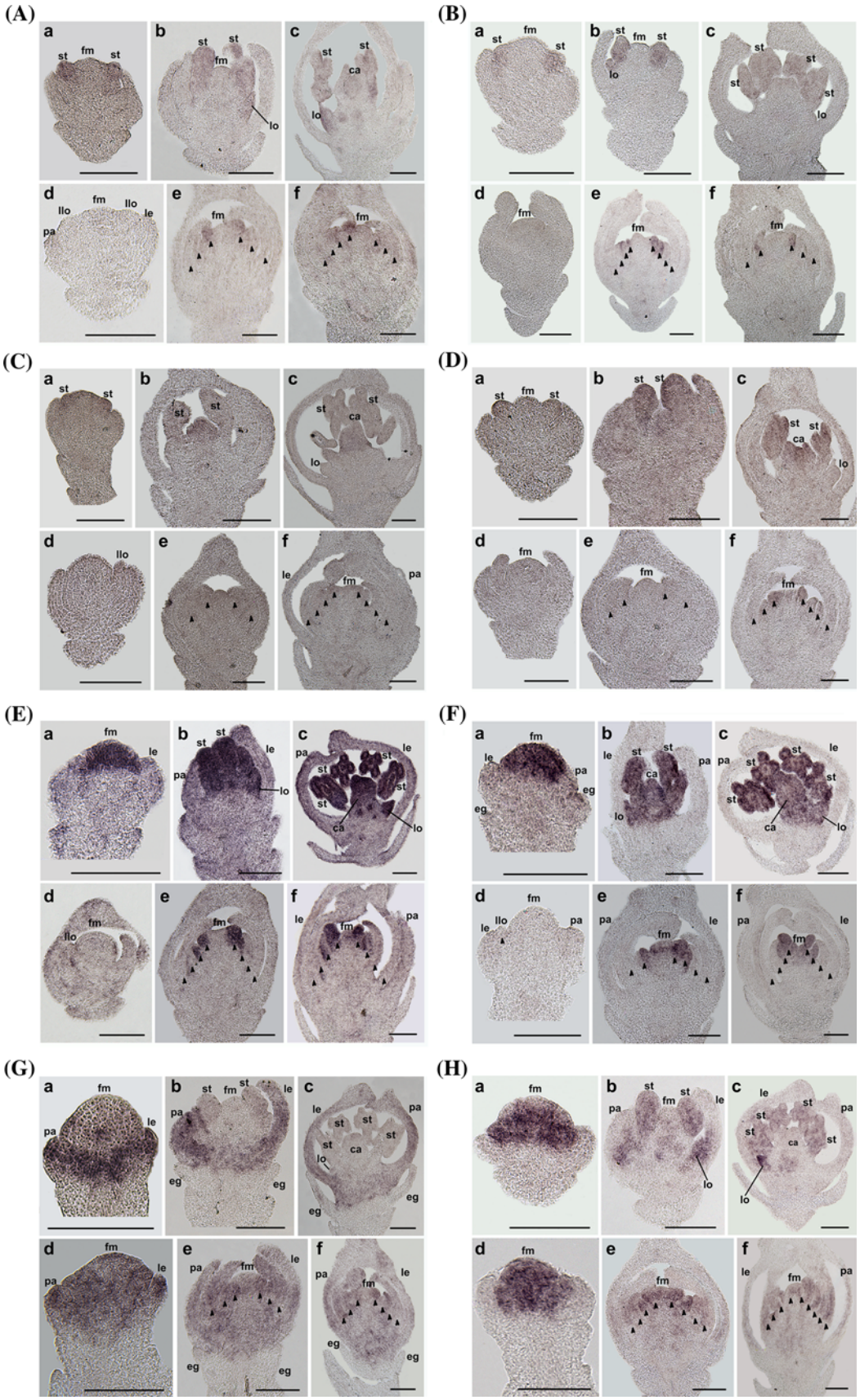
The other *AGL6*-like gene, *OsMADS17*, was expressed intensely in the floral meristem when lemma and palea were initiated in wild-type spikelets. Subsequently, the signal was detected in the lemma, palea, lodicules, stamens and ovule (Fig. 6H[a–c]; Ohmori et al. 2009). In *Osmads6-5*, the early expression level and domain of *OsMADS17* were not significantly altered (Fig. 6H[d]). As the development proceeded, the signal of *OsMADS17* expression appeared in all whorls of LLOs from their initiation. Consistent with the fact that there are more whorls formed in *Osmads6-5*, *OsMADS17* had an increased expression level in the mutant compared with the wild type (Fig. 6H[e, f]).

In summary, the temporal-spatial expression patterns of the floral organ identity genes examined were significantly altered in *Osmads6-5*, supporting the notion that *OsMADS6* is required for their correct spatial and temporal expression.

Discussions

The possible cause of the phenotypic variations of O*sMADS6* alleles

Recent studies have shown that *AGL6*-clade genes are a subfamily of floral MADS-box genes (Becker and Theissen 2003), and significant effects of *AGL6*-clade genes on floral development have been found in monocots but not in dicots (Schauer et al. 2007, 2008; Rijpkema et al. 2009; Thompson et al. 2009; Ohmori et al. 2009; Li et al. 2010; Zhang et al. 2010). In rice, the *AGL6*-clade gene *OsMADS6* has been shown to function in floral development based on the phenotypes of its four previously described mutants. The first and second mutants, *mfo1-1* and *mfo1-2*, were caused by an amino acid substitution in the MADS domain and a retrotransposon insertion at the position of 119 bp downstream of the stop codon, respectively (Ohmori et al. 2009); the third mutant *Osmads6-1* resulted from a 4-bp deletion in the seventh exon (Li et al. 2010); and the fourth mutant *Osmads6-2* (initially named as *Osmads6-1*) was generated by a T-DNA insertion in the first intron (Zhang et al. 2010). However, the altered floral phenotypes displayed in these mutants appeared to be inexplicit and incomplete, mainly consisting of mosaic floral organs. This suggests that they are all probably partial loss-of-function mutants. Hence, the complete loss-of-function of *OsMADS6* remained to be further defined.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In this study, we found the fifth mutant allele *Osmads6-5*, caused by a large insertion-deletion mutation with the deletion of the MADS-domain. All *Osmads6-5* spikelets showed that the floral organs except lemma all lose their identities and are transformed into LLOs, and the floral meristem is indeterminate, leading to potentially endless whorls of LLOs developing in the central area. Notably, unlike the four previously reported mutants, no mosaic floral organs were observed in *Osmads6-5* (Fig. 1). As a DNA-binding domain, mutations in the MADS domain should directly affect the function of the MADS genes. In fact, over-expression of the MADS domain of *OsMADS1* produced abnormal floral phenotype, acting as a dominant negative allele, whereas over-expression of *OsMADS1* mutant without the MADS domain showed no phenotypic alteration (Jeon et al. 2000a). We obtained the similar result for *OsMADS6* showing that the over-expression of the C-terminal 477-bp ORF of *Osmads6-5* produced normal flower phenotype. In addition, the truncated OsMADS6:GFP fusion protein redistribution in cell localization supports a key role of the MADS domain (Fig. S3). These results indicated that a mutant lacking the MADS domain would lose the function of MADS box genes because of its dimerization and DNA binding capacity (Mizukami et al. 1996; Krizek et al. 1999). Therefore, *Osmads6-5* is a bona fide null allele of *OsMADS6*, confirming that it plays a key role in rice flower development. This conclusion is also supported by the phenotype of O*sMADS6* over-expression in rice where the number of lodicules, stamens and carpels are increased (Fig. 4), and by the results of in situ RNA hybridization analysis as well (Fig. 6).

It is necessary to point out that *Osmads6-2* was suggested to be a null allele because no expression signal was detected by RT-PCR in its 3′-terminal region (Zhang et al. 2010). However, in light of its incomplete mutant phenotype (Zhang et al. 2010), *Osmads6-2* appears to still maintain a partial function. Since only the 3′-terminal region was examined while the T-DNA was inserted in the first intron in *Osmads6-2*, the evidence provided by the RT-PCR was not sufficient to exclude the possibility whether the whole coding sequence of the gene was not transcribed. It is possible that at least the first exon, which is located upstream to the inserted T-DNA, could be transcribed to some extent. If this is true, the protein sequence translated from the putative *Osmads6-2* transcript would probably still possess a partial function because the MADS domain of *OsMADS6* is just located in the first exon (Fig. 3).

OsMADS6 is a critical regulator for early flower development in rice

The phenotypes of *Osmads6-5* resemble that of the B + C double mutants. The *Arabidopsis ap3/ag* and *pi/ag* consist of indeterminate number of whorls of sepals replacing all other floral organs (Bowma et al. 1989, 1991), and the maize *si1-R/zag1-mum1* shows normal glumes that enclose reiterated lemma/palea-like organs (Ambrose et al. 2000). *AGL6*-clade is sister to *AGL2*-clade (*SEP*-like) and expected to have a similar function to that of *AGL2*-clade. Interestingly, the main features of the *Arabidopsis sep1/2/3* triple mutant, rice *Osmads1* mutant and *OsMADS7/8* double knockdown plants all look similar to *Osmads6-5* except for two distinguishable floral phenotypes between them. Firstly, the central region of *Osmads6-5* floret is filled with whorls of LLOs instead of an additional abnormal floret with an elongated pedicel as in *sep1/2/3* and *Osmads1*. Secondly, several allelic mutants of *OsMADS1* and *OsMADS1/5/7/8*-RNAi knockdown lines showed the primary character of "*leafy hull*", but this phenotype has never been observed in the five allelic mutants of *OsMADS6* (Pelaz et al. 2000; Agrawal et al. 2005; Cui et al. 2010). The morphological similarity and dissimilarity indicates that *OsMADS6* and *SEP*-like genes might have similar but not the identical functions in regulating floral organ specification and the floral meristem determinacy. Indeed, several previous studies have suggested that *AGL6*-clade might play redundant roles with or even resemble *AGL2*-clade in controlling floral development (Rijpkema et al. 2009; Thompson et al. 2009; Ohmori et al. 2009; Liu et al. 2009; Li et al. 2010; Koo et al. 2010).

The phenotype resemblance among *Osmads6-5*, the B + C double mutants and the mutants/knockdown plants of *AGL2*-clade implies that *OsMADS6* might directly regulate the expression of B-/C-class and *AGL2*-clade genes. Indeed, in this study, we have found that the temporal and spatial expression patterns of the class B (*OsMADS4/16*) and C (*OsMADS3/58*) and *SEP*-like (*OsMADS1/7/8*) genes showed significant alterations in *Osmads6-5* (Fig. 5). Similar results were obtained by Li et al. (2011). Together, these results indicate that *OsMADS6* probably acts as an early regulator of floral meristem identity by controlling the expression of floral organ identity genes. Such a regulatory relationship appears to be consistent with the mutant phenotype of *Osmads6-5*. It is possible that the delayed and reduced expression of most of the class B and C and *SEP*-like genes and the increased expression of *OsMADS1* (*SEP*-like) make inner floral organs transformed into LLOs in O*smads6-5*. Hence, *OsMADS6* acts as a critical regulator controlling the early development of inner floral organs via activating the expression of class B and C and *SEP*-like genes.

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