Cell Research (2010) 20:299-313.
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ORIGINAL ARTICLE

The *AGL6*-like gene *OsMADS6* regulates floral organ and meristem identities in rice

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Although *AGAMOUS-LIKE6* (*AGL6*) MADS-box genes are ancient with wide distributions in gymnosperms and angiosperms, their functions remain poorly understood. Here, we show the biological role of the *AGL6*-like gene, *OsMADS6*, in specifying floral organ and meristem identities in rice (*Oryza sativa* L.). *OsMADS6* was strongly expressed in the floral meristem at early stages. Subsequently, *OsMADS6* transcripts were mainly detectable in paleas, lodicules, carpels and the integument of ovule, as well as in the receptacle. Compared to wild type plants, *osmads6* mutants displayed altered palea identity, extra glume-like or mosaic organs, abnormal carpel development and loss of floral meristem determinacy. Strikingly, mutation of a *SEPALLATA* (*SEP*)-like gene, *OsMADS1* (*LHS1*), enhanced the defect of *osmads6* flowers, and no inner floral organs or glume-like structures were observed in whorls 2 and 3 of *osmads1-z osmads6-1* flowers. Furthermore, the *osmads1-z osmads6-1* double mutants developed severely indeterminate floral meristems. Our finding, therefore, suggests that the ancient *OsMADS6* gene is able to specify "floral state" by determining floral organ and meristem identities in monocot crop rice together with *OsMADS1*.

***Keywords*:** rice, *OsMADS6*, *SEP*-like gene, flower organ, meristem, identity

Cell Research (2010) **20**:299-313. doi: 10.1038/cr.2009.143; published online 29 December 2009

Introduction

Plant flower morphological formation is closely associated with changes in the number, expression pattern and interaction of MADS-box genes. Studies in two model eudicot plants *Arabidopsis thaliana* and *Antirrhinum majus* have led to the classic genetic ABC model that explains how three classes of genes (A, B and C) work together to specify floral organ identity [1]. In *Arabidopsis*, A (APETALA1, AP1; APETALA2, AP2) alone determines sepals, A and B (APETALA3, AP3; PISTILLATA, PI) together specify petals, B and C (AGAMOUS, AG) specify stamens and C alone determines the carpel [1]. Later, two additional classes of genes (D and E) are added in the ABC model. D specifies the ovule [2], while E class genes (*SEPALLATA1/2/3/4*, *SEP1/2/3/4*; formerly *AGL2/4/9/3*) determine the identity of all four whorls of floral organs and regulate floral meristem determinacy [3-6]. In *Antirrhinum*, the orthologs of *AP3*, *PI* and *AG* are *DEF*, *GLO* and *PLE*, respectively [7]. Investigations in eudicot *Arabidopsis* and petunia demonstrated that *SEP* genes may redundantly function as key regulators that either control the mRNA expression of other floral homeotic genes [8] or interact with these floral homeotic regulators to specify the identity of each floral whorl and regulate floral meristem determinacy [9-11].

Grass (Poaceae) is one of the largest flowering plant families of angiosperms with ~10 000 species, including many important crops such as rice (*Oryza sativa*), barley (*Hordeum vulgare*) and maize (*Zea mays*) [12-14]. Evolutionary adoptions in organization and structure of grass flowers resulted in their unique shape, which is apparently distinct from those of higher eudicots and even other monocots [15-18]. Each grass spikelet consists of glumes and one to several flowers. Each flower contains characteristic floral organs of the lemma, the palea and lodicules, as well as stamens and pistil(s) [19]. Despite

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Received 21 October 2009; revised 4 November 2009; accepted 4 November 2009; published online 29 December 2009

the economic importance of grass flowers in producing grains, the underlying mechanism of grass floral organ specification still remains poorly understood [18, 19].

In rice, the B class gene *SUPERWOMEN1* (*SPW1* or *OsMADS16*) that is orthologous to *AP3* is crucial for stamen and lodicule specification. *spw1* mutants display homeotic conversion of stamens to carpels and lodicules to palea/lemma-like structures [20]. Two class C genes *OsMADS3* and *OsMADS58* in rice have been shown to play distinct roles in specifying the identity of lodicules, stamens and carpels [21]. *OsMADS13* and *OsMADS21* are grouped as D class genes on the basis of their expression pattern and functional analyses [22]. More interestingly, another C-function gene, *DROOPING LEAF* (*DL*), orthologous to *Arabidopsis* gene, *CRABS CLAW* (*CRC*), encoding a YABBY domain protein in rice has been identified, playing roles in carpel specification, floral meristem determinacy and the antagonistic function with class B genes [23]. Rice has diverse *SEP*-like genes, with at least five members in the genome [24]. Currently, *OsMADS1* (also called *LEAFY HULL STERILE1, LHS1*) is one of the best-characterized *SEP*-like genes in rice, which has been shown to be required for determining the identity of the lemma/palea and the meristem of inner floral organs [25-29].

Although *AGAMOUS-LIKE6* (*AGL6*)-like MADS-box genes are ancient and widely distributed in seed plants [30], the function of *AGL6*-like genes remains largely unknown. In this study, we studied the function of *OsMADS6* in specifying rice floral organ identities and floral meristem determinacy. *OsMADS6* transcripts were observed in young floral meristems, subsequently in the palea, lodicules, the carpel, the ovule and the receptacle. Mutations of *OsMADS6* caused altered floral organ identities with changed palea morphology, homeotic conversion of lodicules and stamens into ectopic glume-like and mosaic structures, as well as loss of floral meristem determinacy. Furthermore, analysis of *osmads1-z odmads6-1* double mutants revealed that *OsMADS6* acts to specify flower patterning in combination with *OsMADS1* in rice.

Results

Isolation and genetic analysis of osmads6 mutants

To elucidate the molecular mechanism underlying rice floral development, we generated a rice mutant population using the *Japonica* subspecies 9522 background (*Oryza sativa* L. ssp. *Japonica*) treated with ^{60}Co γ-ray (280 Gy) and screened for mutants with flower defects [31]. One mutant line displaying flower morphological defects was identified (Figure 1A-1D). This mutant was designated as *osmads6-1*, because our map-based cloning and sequencing analyses revealed a mutation of the putative *OsMADS6* gene in the mutant. Furthermore, we obtained one Tos17 retrotransposon insertion line of *OsMADS6*, called *osmads6-2* (see below). Because *osmads6-2* is a weaker allele of *OsMADS6* compared with *osmads6-1* (see below), we subsequently performed

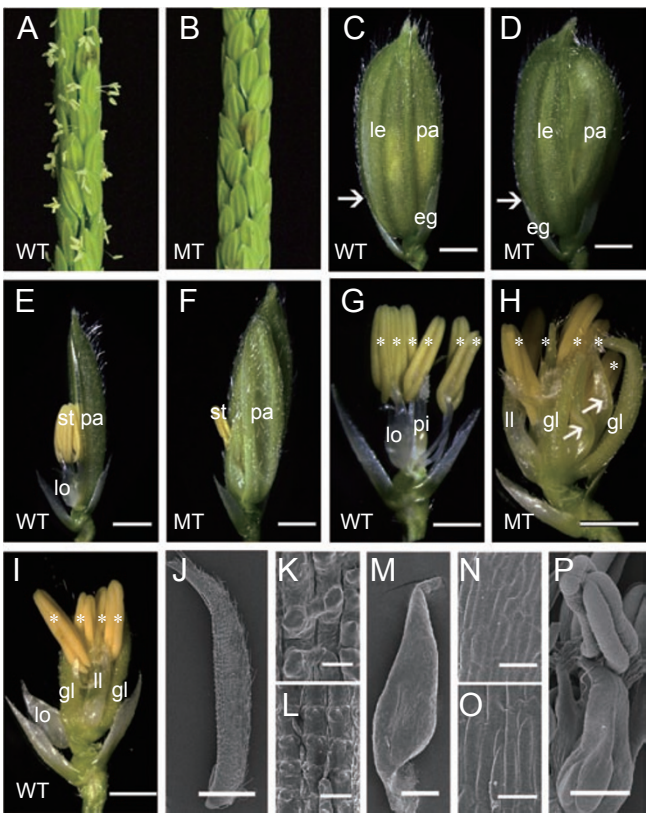

Figure 1 Phenotypes of wild type and *osmads6-1* inflorescences and spiklets. **(A**, **B)** Inflorescences at stage In9 of wild type **(A)** and *osmads6-1* **(B)**. **(C**, **D)** Wild type **(C)** and *osmads6-1* **(D)** spikelets at stage In9 showing enlarged palea in *osmads6-1*. **(E**, **F)** Wild type **(E)** and *osmads6-1* **(F)** spikelets with removed lemmas to show the palea. **(G-I)** Spikelets from wild type **(G)** and *osmads6-1* **(H**, **I)**. The lemma and palea were removed to show inner floral organs. **(J**, **M)** Scanning electron microscopy (SEM) observation of the glume-like organ **(J)** and lodicule-like organ **(M)** in *osmads6-1*. **(K**, **L)** Epidermal cells of wild type palea **(K)** and glume-like organs in *osmads6-1* **(L)**. **(N**, **O)** Outer surfaces of the epidermal cells of wild type lodicule and lodicule-like organs in *osmads6-1*. **(P)** SEM observation of one lodicule-anther mosaic organ. Le, lemma; pa, palea; lo, lodicule; eg, empty glume; st, stamen; ll, lodicule-like organ; gl, glume-like organ; pi, pistil. Asterisk in **G-I** indicates the stamen. Arrows in **H** indicate glume-lodicule mosaic organs. Bars = 1 mm in **C-I**, 500 μm in **J**, 200 μm in **M** and 50 μm in **K**, **L**, **N**, **O** and **P**. Spikelets in **C-I** were at stage Sp8.

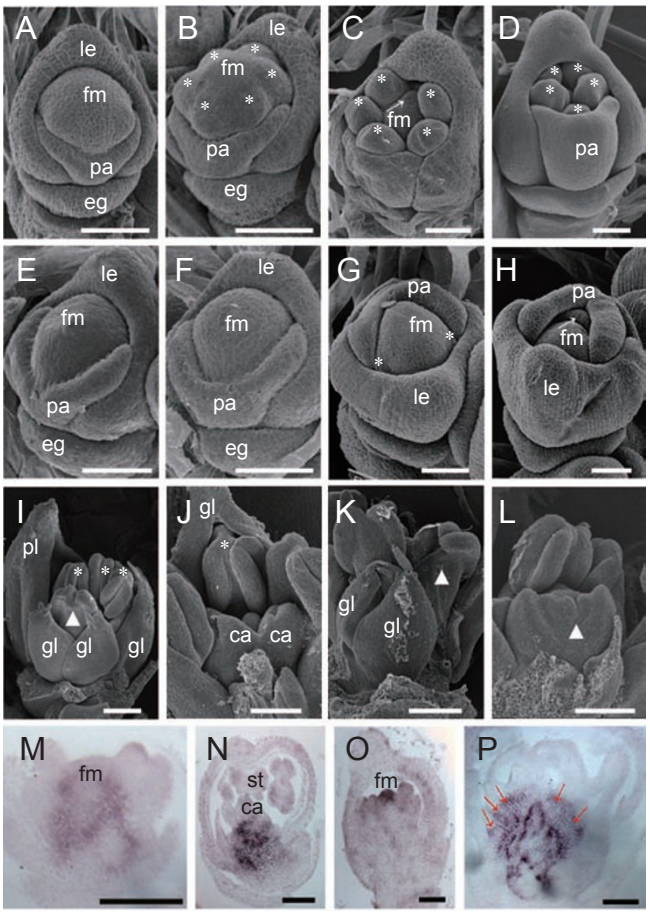

Figure 2 Phenotypes of wild type and *osmads6-1* flowers at early stages. **(A-D)** SEM analysis of wild type flowers at stages Sp4 **(A)**, Sp6 **(B)**, Sp7 **(C)** and Sp8 **(D)**. **(E-H)** SEM analysis of *osmads6-1* flowers at stages Sp4 **(E)**, Sp6 **(F)**, Sp7 **(G)** and Sp8 **(H)**; **(F, G)** showing the delayed initiation of stamen primordia and widened palea and **(H)** showing one glume-like structure. **(I-L)** Inner floral organs of *osmads6-1* at stage Sp7. The lemma and palea have been removed. **(I)** Ectopic glume-like organs enclosing the retarded growth of stamens in the *osmads6-1* flower. **(J)** Two carpel primordia were observed in an *osmads6-1* flower at stage Sp7. **(K)** A flower-like structure with two glume-like structures and one lodicule-anther mosaic organ were observed in an *osmads6-1* flower. **(L)** An *osmads6-1* flower showing indeterminate meristem in the forth whorl. **(M-P)** *OSH1* mRNA signals in the wild type flower at stages Sp6 **(M)** and Sp8 **(N)**. *OSH1* mRNA signals in *osmads6-1* at stages Sp6 **(O)** and Sp8 **(P)**. Le, lemma; pa, palea; lo, lodicule; eg, empty glume; st, stamen; gl, glume-like organ; ca, carpel; fm, floral meristem. Asterisks indicate stamen primordia or stamens, an arrow indicates glume-like organ in **H**, triangular arrows indicate indeterminate structures. Bars = 50 μm in **A-H** and **J-L** and 100 μm in **I** and **M-P**.

more detailed analysis using *osmads6-1*. All of the F1 progenies of *osmads6-1* crossed with wild type showed normal flower development, and an ~3:1 ratio of phenotype segregation was observed in the F2 plants (normal phenotype:mutant phenotype = 352:127; $\chi^2(3:1) = 0.27$, $P > 0.05$), indicating that this mutation most likely oc-

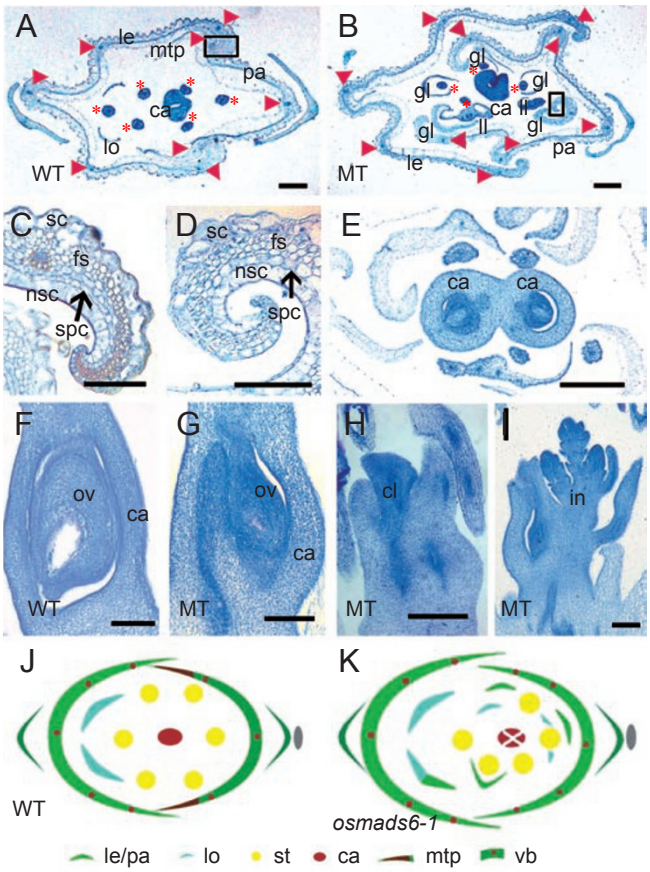

Figure 3 Histological analyses of *osmads6-1* flowers at stage Sp8. **(A)** Transverse sections of a wild type spikelet at the position indicated in Figure 1C by the arrow. **(B)** Transverse sections of an *osmads6-1* spikelet at the position indicated in Figure 1D by the arrow. **(C, D)** Close-ups of the wild type lemma **(C)** indicated in **A** and the glume-like organ **(D)** indicated in **B** to show the four types of cells: silicified cell (sc), fibrous sclerenchyma (fs), spongy parenchymatous cell (spc) and nonsilicified cell (nsc). **(E)** An *osmads6-1* flower with two carpel-like organs. **(F)** A longitudinal section of the wild type carpel showing the ovule structure. **(G)** An *osmads6-1* flower showing the ovule partially transformed into carpel-like organ. **(H)** An *osmads6-1* flower showing one additional ectopic carpel-like organ. **(I)** An additional inflorescence primordium was formed in the center of the *osmads6-1* flower. **(J, K)** Diagrams of wild type and *osmads6-1* spikelets. Eg, empty glume; le, lemma; pa, palea; gl, glume-like organ; ll, lodicule-like organ; mtp, marginal tissue of palea; ov,ovule; sc, silicified cell; fs, fibrous sclerenchyma; spc, spongy parenchymatous cell; nsc, nonsilicified cell. Red arrowheads in **A** and **B** indicate vascular bundles, asterisks in **A** and **B** indicate the stamen. Bars = 100 μm in **A-E** and 200 μm in **F-I**.

curs in a single recessive locus.

osmads6 mutants display altered identities of floral organs and meristem

To characterize the defect of *osmads6* mutants, we performed detailed phenotypic analysis. Compared to wild type, *osmads6-1* mutants showed normal vegetative development and flowering time, and inflorescence morphology of the mutant also appeared normal (Figure 1A and 1B). However, *osmads6-1* mutants displayed defects of floral development compared with wild type (Figure 1C and 1D). During rice flower development, the lemma and palea form at opposite positions on the wild type meristem flank (Figure 2A). Then, two lodicules of whorl 2, which are homologous to the petal in eudicots, are produced interior to the lemma and adjacent to two lemma margins. Six stamens of whorl 3 then emerge in a whorl inside the sterile organs (the lemma, the palea and lodicules) and in the center of the meristem a whorl 4 carpel is eventually formed (Figure 2A-2C). Along the axis of the lemma to palea (Le/Pa), the wild type floral organs display bilateral symmetry, and the lemma and palea form an interlocked structure by their distinctive marginal tissues (Figure 3A) [31]. Although the lemma and palea develop a very similar histological structure comprised of silicified cells, fibrous sclerenchyma, spongy parenchymatous cells and nonsilicified cells [32], the lemma is slightly larger than the palea with characteristic vascular tissue patterns: the lemma has five vascular bundles, whereas three are observed in the palea (Figure 3A and 3C) [31]. In addition, the palea has a distinctive marginal tissue displaying unique smooth epidermis, which lacks epicuticular silicified thickening [31] (Figure 3A and 3C). Compared to wild type, the lemma of *osmads6-1*-mutant flowers was normal (Figures 1C and 1D; 3A and 3B), while the *osmads6-1* palea exhibited widened shape and five to six vascular bundles (Figures 1C-1F; 3A and 3B), resembling the vascular pattern of the wild type lemma (Figure 3A). Moreover, the well-interlocked lemma/palea structure was destroyed in the *osmads6-1*-mutant flower because of the altered marginal tissue (Figures 1E and 1F; 3A-3C).

Unlike two lodicules formed in a wild type flower, the average number of lodicules in the *osmads6-1* flowers decreased to 1.2 (Table 1). In addition, ectopic elongated lodicules or glume-like structures were generated in whorls 2 and 3 (Figure 1H-1I; Table 1). In the third whorl of *osmads6-1*, the average number of stamens decreased to 4.6 (Figure 1H-1I; Table 1). Lodicule-anther mosaic organs with white appearance developed in the mutant (Figure 1H, 1M, 1N, 1O and 1P). Notably, ectopic indeterminate glume-like structures enclosed the stamen filament in *osmads6-1* flowers (Figures 1H, 1I, 1J, 1L; 3B, 3D and 3E). Cellular observation indicated that these ectopic glume-like organs had lemma/palea-like cellular patterns, with silicified cells, fibrous sclerenchyma, spongy parenchymatous cells and nonsilicified cells (Figure 3C and 3D). The wild type ovule is covered by both inner and outer integuments during the late stage (Figure 3F), whereas the *osmads6-1* flower ovule development seemed defective, and the mutant occasionally developed two independent or fused carpels (Figure 3E, 3G and 3H). Moreover, additional inflorescence primordium was observed within the *osmads6-1* flower center in rare cases (Figure 3I). As a result, *osmads6-1* plants displayed fewer seed settings (6%, 64/1161). Also, the defect of ovule development of *osmads6-1* plants was further confirmed by the observation of no seed setting

Table 1 Number of floral organs in whorls 2, 3 and 4 of wild type and *osmads6-1* plants

No. of organs	Glume-like organs*		Lodicule or lodicule-like organs※		Stamens		Lodicule-like or lodicule-anther mosaic organs§		Carpel	
	WT	*osmads6-1*	WT	*osmads6-1*	WT	*osmads6-1*	WT	*osmads6-1*	WT	*osmads6-1*
0	–	–	–	16	–	–	–	6	–	–
1	–	4	–	40	–	–	–	40	70	70
2	–	16	70	19	–	2	–	27	–	9
3	–	37	–	3	–	4	–	4	–	–
4	–	16	–	1	–	27	–	2	–	–
5	–	5	–	–	–	40	–	–	–	–
6	–	1	–	–	70	6	–	–	–	–

A total of 70 wild type and 79 *osmads6-1* spikelets were examined.

*Lemma and palea in first whorl are not included.

※Lodicule or lodicule-like organs present outerside stamens.

§Lodicule-like or lodicule-anther mosaic organs in the whorl of stamens are calculated.

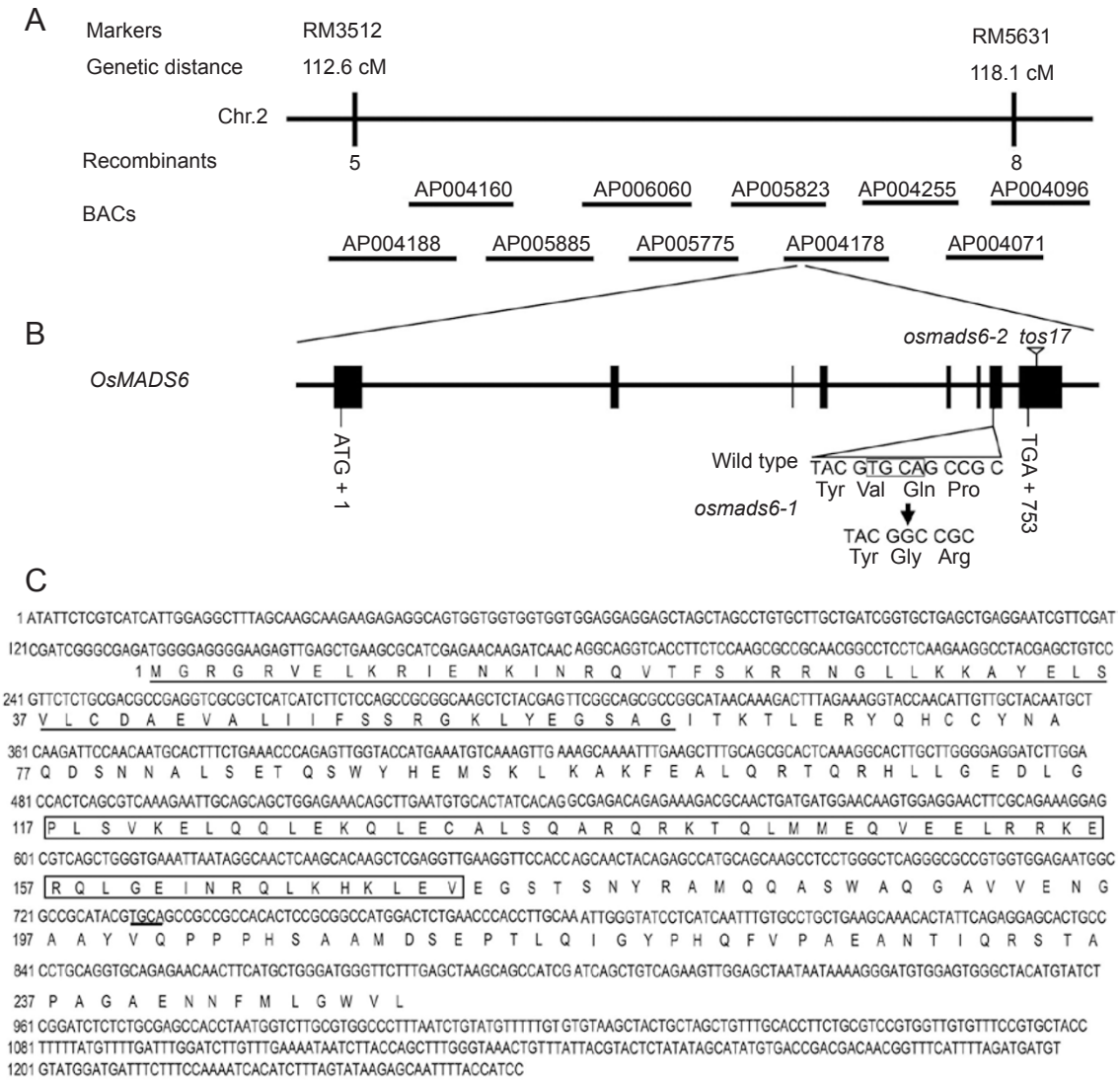

Figure 4 Positional cloning of *OsMADS6* and sequence analysis. **(A)** Positional gene cloning, and the candidate gene was mapped in BAC AP004178 on Chromosome 2. **(B)** Schematic representation of *OsMADS6* and mutations of *osmads6-1* and *osmads6-2*. **(C)** Nucleotide and amino acid sequences of the full cDNA of *OsMADS6*. The MADS-box is showed by underline and the rectangle part represents the K-domain. The fragment between MADS-box and K-box is I-domain and the downstream region of K-box is the C-terminal end.

when mutant plants were crossed with wild type pollen grains. These results suggest that *OsMADS6* is required for specifying floral organs and floral meristem determinacy in rice.

osmads6-1 shows abnormal early development of floral organs

To further characterize the developmental defects of the *osmads6* flowers, scanning electron microscopy (SEM) analysis was performed. To be in line with previous observations, we used the developmental stages defined by Ikeda *et al*. [33]. Till stage Sp4, the primordia of the lemma/palea and empty glumes looked the same in wild type and *osmads6-1* floral primordia (Figure 2A and 2E). During stages Sp6 and Sp7, the wild type flower forms six spherical stamen primordia (Figure 2B and 2C), while *osmads6-1* showed delayed stamen development (Figure 2F and 2G). Subsequently, at stage Sp8 during the formation of carpel primordia in wild type flowers (Figure 2D), glume-like structures around stamen primordia were observed in *osmads6-1* (Figure 2H and 2I). At stage Sp8, with formation of the ovule and pollen grains, wild type flower undergoes normal development of the lemma, the palea, lodicules, stamens and

the carpel, while the *osmads6-1* flower displayed two carpel primordia (Figure 2J), mosaic structures (Figure 2K) and indeterminate floral meristems (Figure 2L). All these observations indicate that *osmads6-1* plants display altered floral organ identities and loss of floral meristem determinacy.

To obtain more evidence for the loss of determinacy in *osmads6-1* floral meristems, the expression pattern of *OSH1* was compared between wild type and *osmads6-1*. *OSH1*, which belongs to the class I knox gene family, is a molecular marker of rice meristematic indeterminate cells [34]. In wild type flowers, *OSH1* transcripts are detected in the floral meristem and receptacle (Figure 2M and 2N). However, the expression domain of *OSH1* became expanded in *osmads6-1* floral primordia, as compared with that in wild type (Figure 2O and 2P), confirming that floral meristems in *osmads6-1* are indeterminate.

Map-based cloning and identification of OSMADS6

To identify the mutant gene, the map-based cloning strategy was used. The homozygous mutant in 9 522 background (a cultivar of *Japonica*) was crossed with one indica cultivar Guangluai. Using a population of 206 F2 mutant plants, the mutation site was mapped between two simple sequence repeat molecular marks, RM3512 and RM5631, on the long arm of rice chromosome 2 (Figure 4A). Within this 926-kb region there is a putative MADS-box gene, *OsMADS6* (os02g45770), annotated in NCBI and TIGR. We hypothesized that *OsMADS6* is likely the candidate gene related to the mutation. By sequencing the RT-PCR product of *OsMADS6* in the mutant, we detected a 4-basepair deletion in the seventh exon of *OsMADS6* (Figure 4B), which causes a frame shift and abnormal translational termination of the C-terminus of the protein (Figure 4B).

Consistently, a mutant line (NE4011) with Tos17 retrotransposon insertion in the 3′-UTR (untranslated region) of *OsMADS6* called *osmads6-2* (Figure 4B) was obtained from Rice Genomic Research Center (RGRC). Overall, *osmads6-2* plants showed similar flower defects as *osmads6-1*. But the phenotype of *osmads6-2* flowers was weaker than that of *osmads6-1* (Supplementary information, Figure S1). *osmads6-2* flowers display abnormal enlarged paleas (Supplementary information, Figure S1). In total, 72% flowers displayed elongated lodicules, and most of the flowers produced one or more ectopic glume-like or elongated lodicule-like organs (n = 37). In addition, 56% flowers generated six normal stamens, while the rest showed changed stamen number. Furthermore, our allelic analysis revealed that F1 plants of the hybrid of *osmads6-1* and *osmads6-2* displayed the mutant phenotype (Supplementary information, Figure S1E-S1H), suggesting that *osmads6-1* is allelic to *osmads6-2*.

The *Os02g45770* was further confirmed to be *OsMADS6* by a double-stranded RNA interference (dsRNAi) experiment using a specific DNA fragment (430-915 bp) of the 3′ region of *OsMADS6* (Supplementary information, Figure S2A). A total of 17 transgenic plants were obtained, among which 9 plants displayed similar floral defects as in *osmads6* mutants (Supplementary information, Figure S2B-S2H). To verify the gene structure of the *OsMADS6* gene, we determined exon-intron junctions by comparing the cDNA clone AK069103 with the genomic sequence, and revealed that *OsMADS6* contains eight exons and seven introns (Figure 4B). *OsMADS6* encodes a putative MADS-box transcription factor with 250 amino acids (Figure 4C).

OsMADS6 belongs to an ancient and conserved AGL6 family shared among gymnosperms and angiosperms

To understand the evolutionary relationship of *AGL6*-like genes, we used the full-length OsMADS6 protein as the query to search for its closest relatives in EST databases from available databases (see methods). Totally, 82 *AGL6*-like genes from gymnosperms, basal eudicots, core eudicots and basal angiosperms were obtained (Supplementary information, Table S1). We then performed phylogenetic analyses on the nucleotide sequences of these genes using both Maximum Likelihood (ML) and Bayesian methods, and 10 *SEP*- and 7 *AP1*-like genes were used as the outgroups (Figure 5; Supplementary information, Figure S3). The phylogenetic tree showed that all *AGL6*-like genes form a well-supported single clade. Within the clade, *AGL6*-like genes were grouped into two separate groups of gymnosperms and angiosperms with strong supporting values, suggesting one common ancestor of *AGL6*-like genes in gymnosperms and angiosperms.

Since the angiosperm-gymonsperm split, the ancenstral gene has expanded by duplications in both lineages. Each of the gymnosperm species from *Cycadopsida*, *Ginkgopsida*, *Coniferopsida* and *Gnetopsida* has two *AGL6* members falling into two distinct gene lineages, named “Gg1” and “Gg2”, suggesting that a duplication event happened after the split of angiosperms and gymnosperms. The *AGL6*-like genes from the basalmost angiosperms *Amborella trichopoda* and *Nuphar advena* were not grouped into the basalmost position in our phylogenetic tree, instead, they were mixed with magnoliid dicots, which might be because of the fact that fewer sequences from basal angiosperms and nongrass monocots were included.

Our phylogenetic analyses showed that the *AGL6*-like genes from grasses form two paralogous clades. One

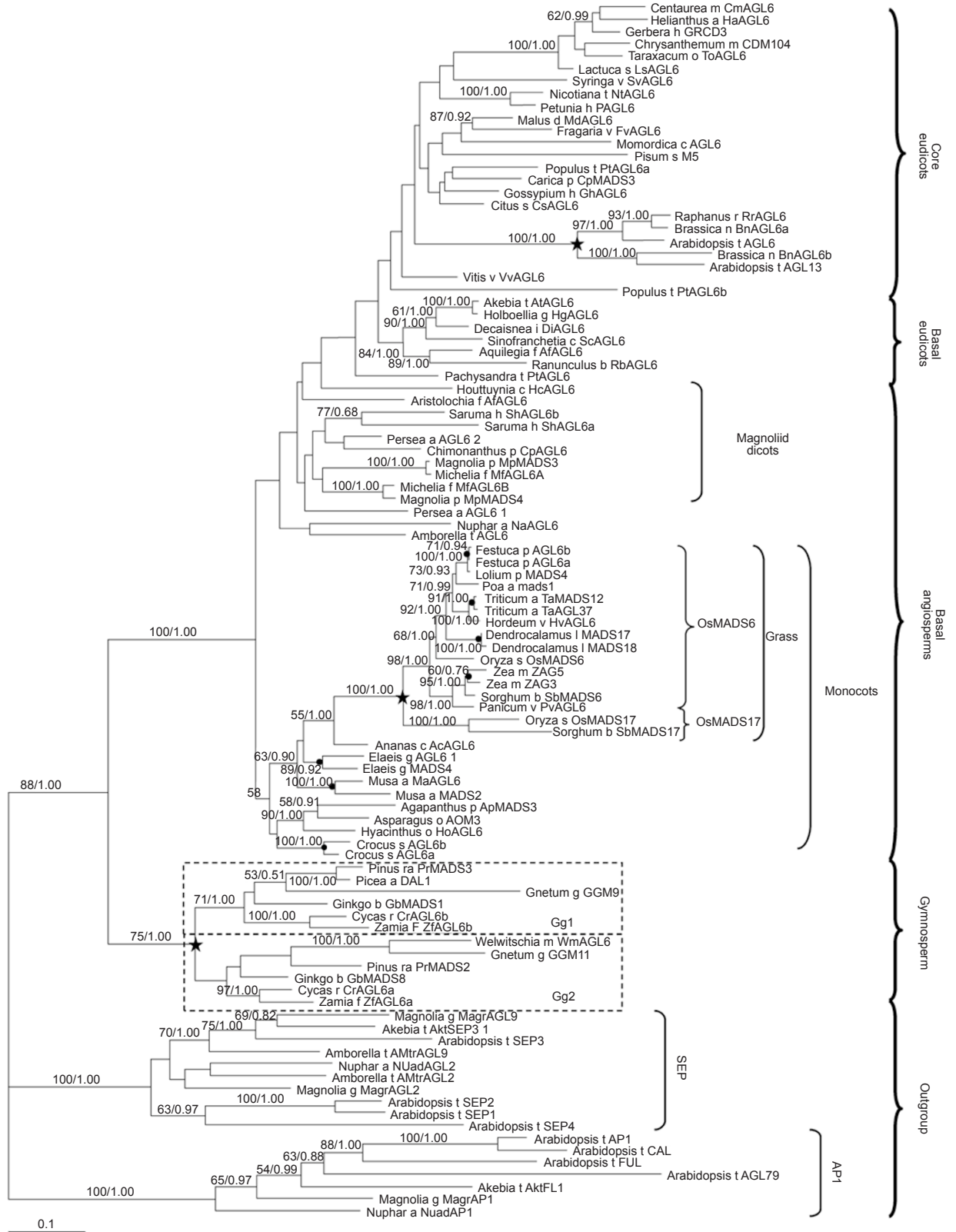

Figure 5 Phylogenetic tree of *AGL6*-like genes with *SEP* and *AP1* genes as an outgroup. The topology of this tree was generated using PhyML. The ML bootstrap values are shown first, followed by the Bayesian posterior probability values. The three major duplication events are highlighted by stars. Small-scale duplication events are indicated with dots. Gg1 and Gg2 are two gymnosperm gene lineages. The scale bar indicates the number of nucleotide substitutions per site.

clade, called *OsMADS17*, contains the *OsMADS17* gene and its putative ortholog from Sorghum, whereas the other, called *OsMADS6*, includes all other genes (Figure 5). This suggests that the duplication event that gives rise to the two clades may have occurred before the diversification of grasses. This is consistent with what has been proposed by Reinheimer and Kellogg [34]. However, our results differ from theirs in that we have included the putative ortholog of *OsMADS17* from Sorghum. In Reinheimer and Kellogg's study, it was suggested that *OsMADS17*-like genes are *Oryza*-specific and may have been lost in all other grasses. In our study, by performing a BLAST search against the Sorghum genome, we identified a genomic sequence that shares very good microsynteny with the region that contains *OsMADS17*. We also noticed that the original annotation for this gene was problematic, as it contained two extra exons that have never been found in *OsMADS17* or other MADS-box genes. Because the original annotation was not supported by EST or cDNA data, we have re-annotated the Sorghum gene according to the exon-intron structure of *OsMADS17* (Supplementary information, Table S1). We found that the *Sorghum* gene, named here as *SbMADS17*, is very similar to *OsMADS17* in sequence features, and thus may be an ortholog of *OsMADS17*.

Moreover, our analysis suggests that recent duplication events of *OsMADS6*-like genes occurred among some species, such as *Triticum aestivum*, *Dendrocalamus latiflorus* and *Zea mays*. Also, similar situation was found in nongrass species, such as *Crocus sativus*, *Elaeis guineensis* and *Musa acuminate*.

AGL6-like genes are closely related to SEP- and AP1-like genes

To further clarify the relationship between *AGL6*-like genes and other MADS-box genes, a total of 33 putative ESTs and annotated protein sequences that are related to rice *OsMADS6* were selected from 22 different species in gymnosperms (7 species) and angiosperms (15 species) (Supplementary information, Table S1). Along with members of *AGL6* sister clades (38 *SEP*-like and 34 *AP1/SQUA*-like genes), these genes were grouped into three well-supported families: *SEP*, *AP1* and *AGL6* (Supplementary information, Figure S3 and Table S1). Seven *TM 3* (tomato MADS-box gene 3)-like, four *AG*-like and four *CRM6*-like MADS-boxes genes were also included as outgroups (Supplementary information, Figure S4).

The topology of the phylogenetic tree suggests multiple gene duplications of *SEP*, *AP1* and *AGL6* members, the first predated gymnosperm-angiosperm split 300 MYA (million years ago), generating *AGL6* and the common ancestor of *AP1* and *SEP*. This hypothesis is also consistent with the molecular clock hypothesis that the diversification of *AGL2/AGL6/SQUA* (or *AP1/AGL9*) clade occurred 373 MYA [35]. However, only *AGL6* subfamily genes have been found in both gymnosperms and angiosperms, while close *AGL6* homologs of SEP and *SQUA* clades have so far been only found in extant angiosperms (Supplementary information, Figure S4) [30, 36]. Either *SEP* and *AP1* genes have been lost during the evolution of gymnosperms or *AGL6*-like genes are basal members compared with clades of angiosperm *SEP*- and *AP1*-like genes. Even though *AGL6*-like MADS-box genes are ancient with wide distributions, the function of *AGL6*-like genes has remained poorly understood. In the *Arabidopsis* genome, there are two *AGL6*-like genes,

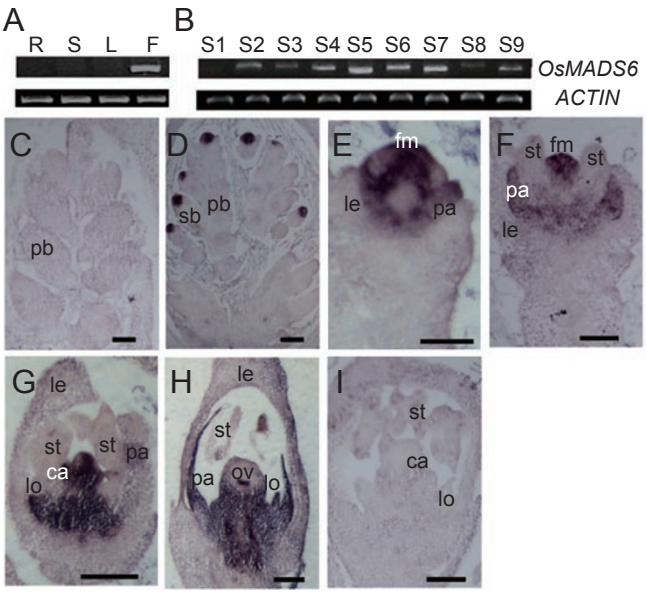

Figure 6 Expression pattern of *OsMADS6*. **(A-B)** RT-PCR analysis showing the *OsMADS6* expression in rice flowers. S1 to S9 indicate stage 1 to stage 9. S1: 0.2 cm inflorescence, S2: 0.5 cm inflorescence, S3: 1 cm inflorescence. S4-S9: represent 1 mm, 2 mm, 3 mm, 5 mm, 7 mm and > 7 mm flowers in length, respectively. **(C-I)** mRNA *in situ* hybridization of *OsMADS6*. **(C)** No detectable signal of *OsMADS6* mRNA in the inflorescence at stage In4. **(D)** Detectable *OsMADS6* mRNA signal in the floral meristem at stage In6. **(E)** Expression of *OsMADS6* in the floral meristem and the palea primordium at stage Sp4. **(F)** Expression of *OsMADS6* in the floral meristem and the palea at stage Sp6. **(G)** Expression of *OsMADS6* strongly in the carpel, lodicules and weakly in the palea at stage Sp7. **(H)** Transcripts of *OsMADS6* in the ovule, lodicules and the palea at stage Sp8. **(I)** A flower at stage Sp8 hybridized with the sense probe of *OsMADS6* as a negative control. R, root; S, stem; L, leaf; F, flower; Pb, primary ranch; sb, secondary branch; Le, lemma; pa, palea; lo, lodicule; eg, empty glume; st, stamen; ca, carpel; ov, ovule; fm, floral meristem. Bars = 100 μm.

AGL6 and *AGL13* (Supplementary information, Figure S4) [37, 38], which are quite closely related and may be formed by a relatively recent gene duplication. However, these two genes display quite distinct expression patterns. The transcripts of *AGL6* were detectable in all four whorls of floral organs [37], whereas *AGL13* was expressed in ovules [38]. The monocotyledonous species maize has two *AGL6*-like genes, *ZAG3* and *ZAG5* [39]. *ZAG3* was expressed in both male and female maize inflorescences [40]. Its expression was found in carpels, but not in stamens, and in the sterile floral organs, but not in glumes [39]. The expression of *Gnetum GGM9* and *GGM11* genes was observed in male and female reproductive cones, but not in vegetative leaves [41]. Expression of *AGL6*-like genes in the reproductive organs appears to be a common status and ancestral trait. All these observations suggest that *AGL6*-like genes likely play an essential and conserved function in specifying plant reproductive organ development.

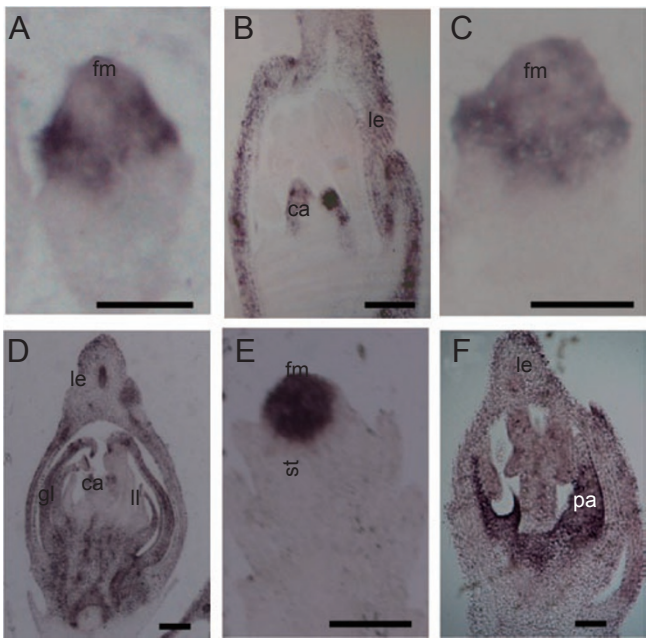

Figure 7 Expression pattern of *OsMADS1* and *OsMADS6* in the mutants. **(A)** Transcripts of *OsMADS1* in the floral meristem of wild type after stage Sp2. **(B)** Transcripts of *OsMADS1* in the lemma and palea, as well as in the carpel primordia at stage Sp7. **(C)** *OsMADS1* transcripts were detected in the floral meristem at stage Sp2 in *osmads6-1*. **(D)** After formation of glume-like structures in *osmad6-1*, *OsMADS1* transcripts were observed in ectopic lemma and palea. **(E)** Expression of *OsMADS6* in the floral meristem of *osmads1-z* at stage Sp4. **(F)** *OsMADS6* is expressed in plaea and leaf or glume-like organ in *osmads1-z* at stage Sp7. Le, lemma; pa, palea; lo, lodicule; st, stamen; ll, lodicule-like organ; fm, floral meristem. Bars = 100 μm.

Expression pattern of OsMADS6

The *osmas6* mutations affected the floral organ development, but had little effect on rice vegetative growth and inflorescence development. To test whether *OsMADS6* acts within the floral organs or from a distant tissue, we analyzed the expression pattern of *OsMADS6*. We first detected *OsMADS6* expression by RT-PCR using total RNA extracted from vegetative and reproductive organs. *OsMADS6* was found to be exclusively and strongly expressed in flowers (Figure 6A and 6B).

To more precisely determine the spatial and temporal patterns of *OsMADS6* expression in floral organs, RNA *in situ* hybridization was performed using an *OsMADS6* antisense RNA probe. Consistent with the result of RT-PCR analysis, the signal of *OsMADS6* transcripts was not detectable at the primary branch primordia at stage In4 (Figure 6C). At stage In6, after the initiation of lemma and palea primordia, *OsMADS6* expression was uniformly detected in floral meristems (Figure 6D) and palea primordia (Figure 6E). Then, the expression of *OsMADS6* was detectable in the palea primordia and flower meristem at stage Sp6 (Figure 6F). During the initiation of lodicules, the *OsMADS6* transcripts were strongly accumulated in the floral meristems, primordia of lodicules and the carpel, but expressed weakly in the palea (Figure 6G). Then *OsMADS6* expression signal was observed strongly in the palea, lodicules, the inner integument of the ovule and weakly in stamens (Figure 6H). Therefore, our data suggest that *OsMADS6* expression is associated with the specification of floral organ and meristem identity.

Expression analysis of OsMADS1 and OsMADS6 in the mutants

OsMADS1 has been identified as a key regulator of floral organs, and the loss-of-function mutant of *OsMADS1*, *lhs1*, develops leafy lemmas/paleas, glume-like structures in whorls 2 and 3, a reduced number of stamens and an increased number of carpels [25]. To address whether the defect in osmads6 is caused by alteration of *OsMADS1*, we analyzed the expression pattern of *OsMADS1* in *osmads6-1* by *in situ* hybridization. Consistent with previous reports [26, 27, 42], at early stages, the *OsMADS1* expression was detectable in the wild type flower meristem (Figure 7A) and subsequently in the lemma/palea and carpel primordia (Figure 7B), but disappeared in the lodicule and stamen primordia (Figure 7B). In the *osmads6-1* mutant, there was no obvious change in *OsMADS1* expression in the spikelet meristem at early stages (Figure

7C), and later in the lemma/palea and carpel (Figure 7D). However, *OsMADS1* transcripts were detectable in the ectopic glume-like organs (Figure 7D), confirming that these ectopic glume-like organs have the characteristics of the lemma/palea. We identified a new null mutant of *OsMADS1* (called *osmads1-z*) with a 1.312-kb deletion in the region spanning the first exon and the first intron of *OsMADS1*. This mutant was confirmed to be allelic to the reported *OsMADS1* mutant *naked seed rice* (*nsr*) (Gao *et al*., submitted and unpublished data) [28]. *OsMADS6* was expressed in the apical region in floral meristems (Figure 7E) of *osmads1-z*, which was quite similar to that in wild type plants (Figure 6D). Later, *OsMADS6* expression was detectable in lodicules, paleas and receptacles. These results indicate that *OsMADS1* and *OsMADS6* do not directly affect each other's expression.

Genetic interaction between OsMADS6 and OsMADS1

Subsequently, we tested whether *OsMADS6* has a genetic interaction with *OsMADS1* in specifying the floral organ development in rice. Compared to the single mutants (Figure 8A, 8B, 8L and 8M), *osmads6-1 osmads1-z* displayed various severe defects in floral meristems and organs. Type I flowers of *osmads6-1 osmads1-z* developed more glume-like organs instead of inner organs inside the lemma and palea (Figure 8C, 8D, 8N and 8O), and one additional spikelet-like organ with empty glumes in the flower center (Figure 8E). Type II flowers of *osmads6-1 osmads1-z* displayed aborted floral organs of whorls 2, 3 and 4, with the lemma and palea enclosing undifferentiated structures or indeterminate meristem in the center of the flowers (Figure 8F-8K, 8P and 8Q). These results suggest that *OsMADS6* specifies the floral organ patterning together with *OsMADS1* (Figure 8M, 8O and 8Q).

Discussion

In this study, we have characterized the function of an ancient *AGL6*-like gene, *OsMADS6*, in specifying rice floral organ and meristem identities. Mutations of *OsMADS6* cause altered flower morphology with altered palea identity, homeotic transformation of lodicules and stamens into glume-like or mosaic organs and occasionally abnormal carpel development. Also, we have shown

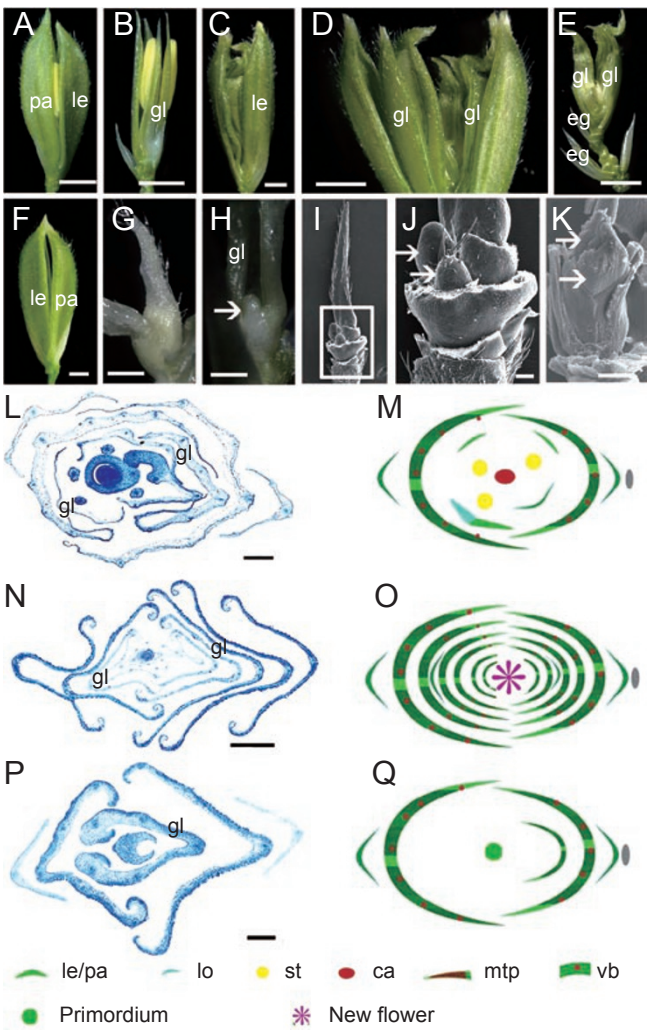

Figure 8 Phenotypes of *osmads1-z* and *osmads6-1 osmads1-z* at stage Sp8. **(A-B)** Flower phenotypes of *osamds1-z*. The lemma and the palea have been removed in **B**. **(C-E)** Type I flower of *osmads6-1 osmads1-z* at stage Sp8. **(E)** The lemma/palea and glume-like organs were removed, two show ectopic glumes and additional flower generated at the carpel position of *osmads6-1 osmads1-z*. **(F-J)** Type II floral phenotypes of *osmads6-1 osmads1-z* at stage Sp8. **(F)** An *osmads6-1 osmads1-z* flower has only one lemma and one palea. **(G)** The lemma and the palea were removed to show inner undifferentiated organs of an *osmads6-1 osmads1-z* flower at stage Sp8. **(H)** Between the two undifferentiated structures, one indeterminate meristem is observable in an *osmads6-1 osmads1-z* flower at stage Sp8. **(I)** SEM observation of the undifferentiated organ with two additional primordia of an *osmads6-1 osmads1-z* flower at stage Sp8. **(J)** Close-up observation in **(I)**. **(K)** One additional developing flower consisting of glume-like organ primordia at the carpel position of an *osmads6-1 osmads1-z* flower. **(L)** Transverse section of an *osmads1-z* flower at stage Sp8. **(M)** The diagram of an *osmads1-z* flower. **(N)** Transverse section of type I defect of an *osmads6-1 osmads1-z* flower. **(O)** The diagram of type I defect of an *osmads6-1 osmads1-z* flower. **(P)** The transverse section of type II defect of an *osmads6-1 osmads1-z* flower. **(Q)** The diagram of type II defect of an *osmads6-1 osmads1-z* flower. Arrow in **H** indicates the meristem, that in **J** indicates organ primodia and that in **K** indicates primodia of GLUME-like organs. Bars = 1 mm in **A-I**, 100 μm in **J**, **K**, **L**, **N** and **P**.

that *OsMADS6* and *OsMADS1* cooperatively define the 'floral state' in rice. These results therefore extend our understanding of the origin and diversification of floral MADS-box genes that contribute to the morphological innovations of grass flowers.

OsMADS6 is a key regulator specifying floral organ identities

Grasses including rice, wheat and maize, produce grains from their flowers, which are the staple food for human beings. Each rice spikelet consists of a flower with one pistil, six stamens and two lodicules subtended by an inner bract or prophyll, called as the palea, and an outer bract called the lemma. In addition, each spikelet contains two highly reduced leaf-like rudimentary glumes at the base in a distichous pattern, and two depressed empty glumes at a position opposite to each other above the rudimentary glumes [33]. Here, we show that *OsMADS6* is a key regulator that controls the development of rice flower organs, but does not affect the development of the lemma, empty glumes and rudimentary glumes. Recently, Ohmori *et al*. [43] reported the identification of two mutants of *OsMADS6* called *mosaic floral organ1-1* (*mfo1-1*) and *mfo1-2*. In *mfo1-1*, the mutation results in a change of Arg to His (R24H) in the MADS domain of OsMADS6 [43]. *mfo1-2* is the same allele as *osmads6-2*, which was identified from a population of Tos17 retrotransposon insertion mutants. Overall, *osmads6-1* shows slightly weaker floral organ defects than those of *mfo1-1* [43].

MADS-box proteins are able to form protein-protein complexes, which are required for their function [9, 44, 45]. In rice, the B class gene *SPW1* (*OsMADS16*) [20] and the C class genes *OsMADS3* and *OsMADS58* [21], as well as the D class gene *OsMADS13* play critical roles in specifying inner floral organ and meristem identities. *OsMADS1/LHS1* has been shown to be a *SEP*-like gene that is required for determining identity of the lemma/palea and the meristem of inner floral organs [25-29]. It is possible that OsMADS6 interacts with other MADS-box protein(s) controlling rice flower development. In agreement with this hypothesis, *OsMADS6* interacts with four *SEP*-like genes (*OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8*), a B class gene (*OsMADS16*) and a D class gene (*OsMADS13*) in yeast cells [46-48]. Further genetic and biochemical analyses of the interaction of these genes with *OsMADS6* will help to elucidate the mechanism of rice flower development.

OsMADS6 plays an important role in specifying rice palea identity

Although recent investigations have identified genes that regulate the lemma/palea development in rice, little is known about the molecular mechanisms of palea development. We have recently shown that the CYCLOIDEA (CYC)-like factor, RETARDED PALEA1 (REP1), is able to regulate palea identity and floral asymmetry in rice [32]. The *REP1* gene is only expressed in palea primordium during early flower development [32]. The *rep1* mutant displays retarded palea development with five vascular bundles at least partially affecting the floral bilateral asymmetric morphology. This finding therefore extends the function of the TCP gene family members in defining the diversification of floral morphology in grasses, and suggests that a common conserved mechanism controlling floral zygomorphy by *CYC*-like genes exists in both eudicots and monocots [32]. In addition, the *palealess* mutant (*pal*) with two leafy structures instead of the palea has been identified [49], suggesting that *PAL1* is another key regulator of palea development in rice. Here, we show that *OsMADS6* is required for the palea identity. *osmads6-1* flowers display changed identity of the palea. Therefore, it will be interesting to learn the relationship among *OsMADS6*, *PAL1* and *REP1* in specifying rice palea development. More recently, investigations on the molecular evolution and expression of the grass *AGL6*-like genes have suggested that expression in the inner integument of the ovule is likely an ancient expression pattern in seed plants, but expression in the palea might reflect a new expression domain in grasses [50]. Therefore, further investigation of the role of *OsMADS6* in palea development may help gain more insights into the mechanisms of grass flower formation.

OsMADS6 specifies 'floral state' together with OsMADS1

OsMADS1 is a *SEP*-like gene, which likely shares the common ancestor with *OsMADS6*. Obviously *OsMADS6* plays a distinct role in specifying floral organ development from *OsMADS1* in rice. Mutations or knockdown of *OsMADS1* in rice result in elongated leafy lemma/palea, glume-like organs, a decreased number of stamens, and occasionally, extra pistils or florets [25-29], while mutations of *OsMADS6* cause the homeotic transformation of paleas to lemma-like organs, lodicules and stamens into glume-like or mosaic structures. *OsMADS6* and *OsMADS1* are both expressed in young floral meristems. *OsMADS6* is subsequently expressed mainly in lodicules, the palea and the ovule. These observations suggest that *OsMADS1* and *OsMADS6* likely play both cooperative and independent roles in regulating floral organ identity. Consistent with this hypothesis, we observed more striking phenotypes in *osmads1-z osmads6-1*. The floral organs of the whorls 2 and 3 in *osmads1-z osmads6-1*

are transformed into glume-like organs or completely repressed during flower development. In addition, *osmads1-z osmads6-1* flowers displayed more severe defect of loss of meristem determinacy. Recently, Ohmori *et al*. [43] reported that the *mfo1-2 lhs1-2* double mutants displayed severe and variable spikelet phenotypes, including extra spikelet(s) without inner floral organs, extra glume-like organs without inner floral organs or abnormal lemma and palea lacking floral organs. Together, these observations strongly support that *OsMADS1* and *OsMADS6* act to specify the 'flower state' in rice.

AGL6 genes regulate flower patterning

Although *OsMADS6* is closely related to *Arabidopsis AGL6*, their functional roles were poorly understood. Our analysis along with previous investigations suggests that *SEP*- and *AP1*-like genes in angiosperms were originated from the common ancestor of *AGL6*-like gene by two duplication events [10]. In addition, another duplication event of *AGL6*-like genes likely occurred before the origin of grasses. Poaceae has two paralogous clades, *OsMADS6* and *OsMADS17*. These observations suggest that *AGL6* genes may have an ancient and conserved role in flower development during evolution.

Consistently, several recent pieces of evidences have shown that *AGL6*-like genes are critical for flower development in several angiosperms. Overexpression of *AGL6* orthologs from orchid (*Oncidium Gower Ramsey*) [51] and from hyacinth (*Hyacinthus orientalis*) [52] has been shown to promote flowering and cause flower homeotic transformation in *Arabidopsis*. Rijpkema *et al*. [53] have recently revealed that the *Petunia hybrida AGL6* (*PhAGL6*, formerly called *PETUNIA MADS-BOX GENE4/pMADS4*) gene functions redundantly with the *SEP* genes, *FLORAL BINDING PROTEIN2* (*FBP2*) and *FBP5*, in specifying petal and anther development. Reinheimer and Kellogg [50] have analyzed the molecular evolution and expression pattern of the grass *AGL6*-like genes. They hypothesize that the expression of *AGL6*-like genes in the inner integument of the ovule seems to be an ancient expression pattern, which is closely related to the expression of the *AGL6*-like genes in the megasporangium and integument in gymnosperms [50]. On the other hand, angiosperms have acquired the new expression pattern in floral meristems and in second whorl organs in monocots [50]. Furthermore, a maize *AGL6*-like gene *bearded-ear* (*bde*) has been recently shown to play pleiotropic roles in controlling floral meristem determinacy, organ specification and sex determination [40]. The *bde* mutant displays abnormal floral development; extra floral mosaic organs are observed in the upper floral meristem, but the lower part develops additional floral meristems [40].

AGL6-like genes have a SEP-like role in flower patterning

Studies on the grasses suggest that the functions of B and C class genes are conserved in plants, but the roles of A and E class genes remain largely unknown in grass plants. Together with our finding, we propose that *OsMADS6* plays a very similar functional role to those of the *SEP* clade (E class genes). *OsMADS6* regulates the development of all four whorls of floral organs and the floral meristem determinacy, which is similar to the role of *SEP* genes [5]. *osmads6-1 osmads1-z* double mutant displays more glume-like organs inside the flower, which is similar to *sep1 sep2 sep3* triple mutants that exhibit sepal-like floral organs [5], and *sep1 sep2 sep3 sep4* quadruple mutants that exhibit leaf-like floral organs [6]. In addition, *OsMADS6* has a similar expression pattern to that of *SEP* genes [5, 6]. Furthermore, OsMAS6 is able to form protein complexes with rice B and D proteins in yeast cells [47, 48], resembling the complex formation of SEP proteins with A, B and C in *Arabidopsis* and other eudicot species [9, 54]. Moreover, a physical interaction between BDE and ZAG1 (Zea AGAMOUS 1, a C class protein) in maize has also been shown recently [40].

Although the ABCDE model seems to be partially applicable in explaining how the flower organs are controlled by the regulators in grasses, there are many different aspects in *Arabidopsis* and rice. For instance, recently, we have revealed the biological role of one *SEP*-like gene *OsMADS34* in controlling the development of inflorescences and spikelets in rice (Gao *et al*., submitted and unpublished data). Even though *OsMADS34* is closely related to two *SEP*-like genes, *OsMADS1* and *OsMADS5*, it displays distinct functional and sequence aspects. *OsMADS34* encodes a MADS-box protein with a shortened C-terminus without transcriptional activation activity. *OsMADS34* is ubiquitously expressed in roots, leaves and primordia of inflorescence and spikelet organs (Gao *et al*., submitted and unpublished data). Further genetic and biochemical analyses of MADS-box genes in rice will facilitate our understanding of the flower development in rice.

Material and Methods

Plant materials

The single recessive rice mutant *osmads6-1* showing indeterminate floral organs was identified from M2 population of 9522 (cv. *Japonica*), mutagenized with radiation of γCo^{60}. Another allele of *OsMADS6*, *osmads6-2*, which was verified by allelism test and obtained from RGRC, is one Tos17-insertion mutant line (NE4011). The 9522 cultivar was used as a wild type strain for observation of phenotypes and for RNA *in situ* analysis. All plants were grown in

the paddy field or greenhouse in Shanghai Jiaotong University.

Histological analysis and scanning electron microscopy observation

Materials were fixed in FAA and dehydrated in a series of graded ethanol. For histological analysis, tissues were substituted by xylene and embedded in paraplast plus. Then, materials were sectioned into 8-μm thick sections, stained with toluidine blue and observed using a light microscope. Transverse sections were photographed using a Nikon E600 microscope and a Nikon DXM1200 digital camera. SEM observation was observed with JSM-6360LV (JEOL) scanning electron microscopy, as described previously [55].

In situ hybridization

Treatment of samples was carried out as described previously [55]. Gene-specific fragment at the 3′ end of *OsMADS6* cDNA (430-915 bp) was amplified by RT-PCR and cloned into pBluescript II KS+ phagemid vector (Stratagene). The construct of probes for *OsMADS1* and *OSH1* has been described previously [29, 34]. Digoxygenin-labeled antisense and sense probes of *OsMADS6*, *OsMADS1* and *OSH1* were transcripted *in vitro* as described previously [55]. Images were obtained using the Olympus Nikon E600 microscope.

Transgenic plants

To construct the dsRNAi vector of *OsMADS6*, two copies of the partial cDNA at 3′ end (430-915 bp) amplified by RT-PCR were placed upstream and downstream of a single intron of *GUS* gene in bridge vector in forward and reverse directions. Then, total fragment containing two copies of cDNA and *GUS* intron were inserted into a binary vector containing a cassette of double 35S promoter and rbcs polyA. The recombinant plasmid was transformed into the wild type calli by the *Agrobacterium*-mediated method [56].

Phylogenetic analysis

Data retrieval *AP1*-like genes, *SEP*-like genes and *AGL6*-like genes were retrieved from previously published studies and other publicly available databases using BLAST searches. During the BLAST searches, multiple genes of the each subfamily from different lineages were used as queries. The following databases were used in the search: DFCI (http://compbio.dfci.harvard.edu/tgi/plant.html), JCVI (J. Craig Venter Institute), NCBI (National Center for Biotechnology Information), FGP (http://pgn.cornell.edu/), TGI (http://compbio.dfci.harvard.edu/tgi/plant.html) and PGN (http://pgn.cornell.edu/). Each of the databases was searched using TBLASTN. We obtained the sequences whose *E*-values were below le–5 and redundant sequences with identity of at least 95% were removed from our data set (Supplementary information, Table S1).

Multiple sequence alignments Protein sequences were first aligned with CLUSTALX 1.83 [57]. Sequences of the alignment were ordered according to their phylogenetic placements in the preliminary tree, then, they were aligned manually using GeneDoc (version 2.6.002) software (Pittsburgh Supercomputing Center; http://www.psc.edu/biomed/genedoc/). A DNA version of this alignment was also generated using the publicly available software aa2dna (http://homes.bio.psu.edu/people/Faculty/Nei/Lab/software.htm).

Phylogenetic analyses Phylogenetic analyses about AGL6 were conducted using DNA alignments that included the conserved M-, I- and K-domain regions, while the analysis about *AGL6*, *SEP* and *AP1* were conducted using DNA alignments that included the conserved M-, I- and K-domain regions and the C-terminal residues with higher than 12 quality scores. The quality score for each residue was estimated in CLUSTALX 1.83 [57]. The PhyML software [58] was used to construct ML tree with the most appropriate model, GTR+I+C, which was estimated by running MODELTEST version 3.06 [59] and 1 000 bootstrap replicates. The MrBayes software [60, 61] was used to construct Bayesian trees after running for 10 million generations using 4 Markov chains, and sampled every 100 generations using the GTR+I model. Variation in the likelihood scores to determine apparent stationarity was examined graphically using the program Tracer version 1.2.1 (A Rambaut and A Drummond, University of Oxford, unpublished data). Tree files were viewed using TreeView.

Acknowledgments

We gratefully acknowledge B Han from National Center for Gene Research, Chinese Academy of Sciences (CAS) and Rice Genome Resource Center (RGRC) for providing BAC clone, cDNA clone and Tos17 insertion line. We thank Z-J Luo and M-J Chen from Shanghai Jiao Tong University for mutant screening and generation of F2 populations, X-Y Gao from Institute of Plant Physiology and Ecology, SIBS, CAS, for SEM, H Yu from National University Of Singapore for critical reading of this manuscript and H Ma from Fudan University for helpful discussion. This work was supported by funds from the National Basic Research Program of China (2009CB941500, 2006CB101700), the National Natural Science Foundation of China (30725022, 30830014 and 90717109) and the Shanghai Leading Academic Discipline Project (B205).

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