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Ectopic expression of rice *OsMADS1* reveals a role in specifying the lemma and palea, grass floral organs analogous to sepals

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Abstract MADS-domain-containing transcription factors play diverse roles in plant development. The prototypic members of this gene family are the floral organ identity genes of the model dicotyledonous plant, *Arabidopsis thaliana*. Sequence relatedness and function ascribe them to *AP1/AGL9*, *AG*, *AP3* and *PI* gene groups. The rice MADS-box gene, *OsMADS1*, is a member of the *AP1/ AGL9* sub-group. Tomato and *Petunia* members of this sub-group specify floral meristem identity and control organ development in three inner whorls. Reported here are phylogenetic analyses that show *OsMADS1* to form a distinct clade within the *AGL9* gene family. This sub-group currently has only three other monocot genes. We have studied the expression pattern of *OsMADS1* and determined the consequences of its ectopic expression in transgenic rice plants. *OsMADS1* is not expressed during panicle branching; earliest expression is in spikelet meristems where it is excluded from the outer rudimentary/sterile glumes. During organogenesis, *OsMADS1* expression is confined to the lemma and palea, with weak expression in the carpel. Ectopic *OsMADS1* expression results in stunted panicles with irregularly positioned branches and spikelets. Additionally, in spikelets, the outer rudimentary glumes are transformed to lemma/palea-like organs. Together, these data suggest a distinct role for *OsMADS1* and its monocot relatives in assigning lemma/palea identity.

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Introduction

The shoot apical meristem of higher plants contains undifferentiated progenitor cells that give rise to both vegetative and reproductive structures. Transition from vegetative to reproductive phase is controlled by both genetic and environmental factors and is responsible for specifying floral identity to newly arising meristems. The flowers formed hence are complex reproductive structures wherein the invariant ordering of floral organs – sepals, petals, stamens and carpels from the periphery to the center – implies a common ground plan for organ patterning in all flowering plants. In the model dicot plant *Arabidopsis thaliana*, conferment of floral fate requires the activity of meristem identity genes: *LFY*, *AP1*, *AP2* and *CAL* (Weigel et al. 1992; Bowman et al. 1993; Weigel 1995). In addition, these floral meristem identity genes are transcription activators of floral organ identity genes that pattern organs in four concentric whorls (Parcy et al. 1998; Wagner et al. 1999). The latter genes include *AP1*, *AP2*, *AP3*/*PI* and *AG*, identified first on the basis of loss-of-function homeotic mutations that alter cell fate in two adjacent whorls of the flower (reviewed by Ma 1994; Irish 1999). Thus, their combined and individual domains of action are proposed to dictate organ fate. All but one of these *A. thaliana* floral organ identity genes belong to the MADS-domain-containing group of evolutionarily conserved transcription factors that regulate gene expression in plant, yeast and animal cells (Reichmann and Meyerowitz 1997). The temporal and spatial expression pattern of the floral organ identity genes is consistent with their function in two adjacent whorls (Yanofsky et al. 1990; Jack et al. 1992; Mandel et al. 1992b; Goto and Meyerowtiz 1994). For instance, *AG* is expressed in whorls 3 and 4 of the flower and loss-of-*AG* function results in altered cell fate in these whorls alone (Yanofsky et al. 1990). The sufficiency of these

genes to alter cell fate, when expressed ectopically in floral whorls where they are not normally found, has also been tested (Mandel et al. 1992a; Mizukami and Ma 1992; Jack et al.1994). All of these data are, to a large extent, explained and accommodated by the ABC model for floral organ specification, originally proposed based on the phenotypes of loss-of-function mutations in these floral organ identity genes (Coen and Meyerowitz 1991; Ma 1994; Irish 1999).

The MADS-box genes have provided a gateway to probing the function of regulatory transcription factors in patterning floral organs in diverse species. They also provide tools to study mechanisms by which sequence homologues and/or orthologues could contribute to species-specific differences in floral organ patterns. Modern day monocotyledonous (monocot) plants shared ancestors with dicotyledonous (dicots) plants more than 120 million years ago (Doyle 1973). The divergent and reduced flowers of cereal grasses, a large group among the monocots, provide an excellent model to examine the above mentioned hypotheses. Typical of grasses, rice flowers are borne on a branched inflorescence called the panicle, where the developmental progress from inflorescence specification to flower meristem allocation occurs in steps. Upon floral induction, the inflorescence meristem generates primary branch primordia on its flanks, which in turn elongate and allocate basally second order branch primordia. Subsequently, spikelet primordia are specified apically on the primary as well as on the secondary branch primordia (Hoshikawa 1989). In sum, the inflorescence/panicle meristem, primary branch primordia, secondary branch primordia, and floral primordia comprise the reproductive meristems in rice. A rice spikelet primordium bears a single flower in the axil of rudimentary glumes and the flower contains the organs-lemma, palea, lodicules, stamens and carpel. Recently, several rice MADS-box genes have been partially characterized (Chung et al. 1994, 1995; Kang et al. 1995, 1998; Kang and An 1997). However, their precise role during panicle development remains largely unknown. One such MADS-box gene is *OsMADS1*, whose predicted product has overall 68.4% identity to AGL9, 56.2% identity to AGL2, and 44.4% identity to AP1 proteins of *Arabidopsis* (Chung et al. 1994). All of these *Arabidopsis* genes are initially expressed throughout the floral meristem. Their later expression patterns vary; they are either expressed in all four floral organ primordia, or only in the perianth, or in three inner whorls. Preliminary studies on *OsMADS1* reveal it to be temporally and spatially regulated, with features both similar to and different from *AP1/AGL9*-like factors. Early expression is uniform throughout the floral meristem and its later high level expression is restricted to the lemma and palea (modified first whorl organs), with weak expression in the carpel (Chung et al. 1994; Vijayraghavan 1996). We have examined the phylogenetic relationships of *OsMADS1* and its closely related homologue *OsMADS5*. We find that they form a distinct clade within the *AP1/AGL9* group with no members as yet from any dicot species that can be assigned to this sub-group. Also, we have studied in detail the expression pattern of *OsMADS1* and have determined that it is not expressed in the spikelet organs, i.e. the glumes, which are modified bracts peripheral to the flower. Additionally, by adopting a reverse genetics strategy, we have studied the consequences of ectopic *OsMADS1* expression to understand its role in organ fate specification. We show that *OsMADS1* has a role in assigning cells to a lemma and palea fate, and that its ectopic expression affects panicle branch differentiation.

Materials and methods

Phylogenetic analysis

The nucleotide and protein sequence of *OsMADS1* was retrieved from the GenBank sequence database (Accession Number L34271). The predicted protein from the full-length cDNA sequence was used as query in BLAST and FASTA searches for related sequences in the GenBank and SwissProt non-redundant sequence databases. The high scoring sequences were retrieved from the databases. Plant MADS-box genes used for phylogenetic analysis are given below, with their accession numbers given within brackets. Rice genes, *OsMADS1* (L34271), *OsMADS2* (L37526), *OsMADS3* (L37528), *OsMADS4* (L37527), *OsMADS5* (U78890), *OsMADS6* (U78782), *OsMADS14* (AF058697), *OsMADS15* (AF058698), *OsMADS16* (AF077760), *OsMADS17* (AF109153), *OsMADS45* (U31994), *RAP1b* (AB041020), rice MADS-like (*RML*; (AB003324); *Arabidopsis* genes *AGL2* (B39534), *AGL3* (P29383), *AGL4* (P29384), *AGL6* (M55554), *AGL9* (AF015552), *AGL13* (U20183), *AP1* (Z16421), *AGAMOUS* (S10933); and maize genes *ZAP1* (L46400), *ZAG1* (L18924), *ZAG2* (X80206), *ZMM1* (X81199), *ZMM2* (L81162), *ZMM3* (Y09301), *ZMM8* (Y09308) and *SILKY1* (AF181479) were retrieved from GenBank. The sequences for tomato *TDR5* (X60480), petunia *FBP2* (M91666) and *Antirrhinum PLENA* (S53900) genes were also retrieved from GenBank. Yeast *MCM1* gene (P11746) and human *MEF2* (L16794) gene sequences were also similarly retrieved.

Alignment and sequence analysis

For alignment and sequence analysis the following approaches were taken. The full-length protein sequences were aligned using the PILEUP of the UWGCG package. This was refined using CLUSTALW 1.7. Phylogenetic analysis, with the full-length protein sequence, employing maximum parsimony methods performed using the experimental version of PAUP* program, (4.0.0d55 Version for UNIX; D.L. Swofford, Laboratory of Molecular Systematics, Smithsonian Institute, Washington, D.C). For maximum parsimony analysis 33 taxa were analyzed, with 100 replicates, keeping all optimal trees in each replicate. Gaps were treated as missing residues. Tree construction was undertaken using the Bootstrap method with Neighbor-Joining (NJ) or Heuristic search. The NJ analysis was done using the software default settings and the optimality criterion was set to distance. The trees were examined using the PAUPDISPLAY program of the UWGCG package. The tree obtained using Bootstrap method with heuristic search algorithm was essentially the same as that obtained with NJ. The MCM1 protein sequence was used as outgroup in this analysis, to study the branching order of the gene lineages relative to the main group. In addition, a tree was also generated with the plant floral organ identity gene *AG* as the outgroup. Distance based criteria (Kimura protein distance) and NJ were used to generate the tree.

Furthermore, in the aligned predicted protein sequences, the 525 nucleotides coding for the MADS-intermediate-keratin-like

(MIK) region of OsMADS1 and related proteins were visually selected, manually refined and analyzed. These nucleotide sequences were re-aligned using the PILEUP program. The 120 nucleotides corresponding to the MADS box and 233 nucleotides corresponding to the K box sequences were thus deciphered. Sequence divergence at the nucleotide level was then derived using the DISTANCES program of the UWGCG package. The Tajima and Nei method (Tajima and Nei 1984) was used to estimate rate of nucleotide substitution. The resulting distance matrix was processed using the GROWTREE program of the UWGCG package. The tree was constructed using the NJ method. The trees were visualized with TREEVIEW. The DIVERGE program of the UWGCG package based on the methods described by Li et al. (1985) and Li (1993) was employed to determine the rates of non-synonymous substitution in the MADS box, K box and MIK region.

Isolation of *OsMADS1* cDNA clone

A λcDNA library in the vector λUniZAP (Stratagene), representing mRNAs from wild-type rice panicles (length 2–5 cm; Kushalappa 1999), was screened to obtain full-length *OsMADS1* cDNAs. The probe used was a partial cDNA, previously cloned in the laboratory that encoded amino acid residues 175–257 of the predicted OsMADS1 protein (Chung et al. 1994; Vijayraghavan 1996). From screening 10^6 plaques, 12 positives were obtained, one of which contained the full-length cDNA as determined by sequence analysis of the excised phagemid.

Construction of transgene to ectopically express *OsMADS1*

Binary vector pCAMBIA1300 was modified for ectopic expression of *OsMADS1*. The complete maize ubiquitin (*Ubi*1) promoter (Christensen et al. 1992) was cloned in as a *Hin*dIII-*Bam*HI fragment. Subsequently, the nopaline synthase (*nos*) terminator was cloned as a *Sac*I-*Eco*RI fragment downstream of the *Ubi*1 promoter. Lastly, *OsMADS1* full-length cDNA was blunt-end cloned into the *Sac*I site between the promoter and terminator sequences. The sense orientation of the cDNA was verified by *Kpn*I digestion. The recombinant was called pUbi-*OsMADS1*.

Rice transformation

The embryogenic calli from rice seeds of the *japonica* variety TP309 were co-cultivated with *Agrobacterium tumefaciens* (Hiei et al. 1994) carrying the pUbi-*OsMADS1* plasmid. Co-cultivation was for 2–3 days in the dark (at 25°C) on 2N6-AS medium supplemented with 100 µM acetosyringone. The co-cultivated calli were washed with water containing 250 mg/l cefotaxime and then transferred onto N6 medium containing 50 mg/l hygromycin and 250 mg/l cefotaxime for 8 weeks. Actively proliferating calli were further transferred onto regeneration medium (3 mg/l BAP and 0.5 mg/l NAA) for 4–8 weeks and subsequently onto 1/2 MS medium (0.05 mg/l NAA) for 3 weeks. A light/dark cycle of 16/8 h was provided during regeneration and rooting. Plantlets were then transferred to soil and grown in a greenhouse.

Light microscopy and scanning electron microscopy

Morphological features of transgenic and wild-type panicles and flowers were observed by stereomicroscopy. For SEM, panicles were dissected away from the enveloping leaf sheaths, fixed, dehydrated and then critical point dried. The dried panicles were mounted onto stubs, sputter coated with gold and then viewed in a Joel JSM-5310 LV microscope (Skandinaviska AB, Sundbyerg, Sweden).

RT-PCR of *OsMADS1* transcripts

Total RNA from young leaves of transgenic plants or control plants was isolated by phenol extraction and then treated with RNAse-free DNAse. RT-PCR experiments were performed using this total RNA (Wang and Tobin 1998; Nandi et al. 2000). First strand cDNA was made from 7.5 µg RNA in 20 µl reaction mixture using the primer OSM2-1 (5′ CGGGATCCGCCAATTAATTGTTACC 3′). The primer used in control cDNA synthesis reactions was Act2 (5′ GATGGATCCTCCAATCCAGACACTGTA 3′). Standard PCR reactions of 50 µl were then performed with 5 µl RT product generated for *OsMADS1* or *ACT1*. PCRs contained, as the forward primer, OSM4 (5′ GAGAATGTGCTCCATATG 3′) in the *OsMADS1* RT-PCR or Act1 (5′ GTATTGTGTTGGACTCTGGTGATGGTGT 3′) in the *ACT1* RT-PCR reactions. PCR products were visualized on 1% agarose gel after loading 10 µl from each PCR reaction.

In situ hybridization for *OsMADS1* transcripts

Preparation of rice tissues and hybridization conditions included only minor modifications of the protocol as described by Drews et al. (1991) for *Arabidopsis*. The *OsMADS1* ^{35}S-labeled anti-sense mRNA probe was synthesized with T3 RNA polymerase from a *Bam*HI digest of a plasmid containing 500 bp of the 3′ gene specific region of *OsMADS1*. The slides were exposed for 1 week.

Results and discussion

Phylogenetic relationships of *OsMADS1*

A preliminary sequence alignment of the predicted OsMADS1 protein to a few other plant MADS-domain-containing factors had suggested relatedness to the *AGL2* and *AP1* genes of *Arabidopsis* (Chung et al. 1994). This homology was consistent with its flower-specific expression in the lemma, palea and carpel (Chung et al. 1994; Vijayraghavan 1996). Members of the plant MADS-domain gene family can be distinguished by phylogenetic relationships, and such analyses show them to cluster into distinct groups: *AP3/PI*, *AG*, *AP1/AGL9* and orphan gene clades (Purugganan et al. 1995; Theissen et al. 1996; Alvarez-Buylla et al. 2000). Unlike members of the *AP3* or *PI* groups, the precise function of many of the molecules belonging to the *AP1/AGL9* clades remains unknown. Additionally, this group bears within it multiple lineages, the most obvious being *AP1*, *AGL9* and the *AGL6/AGL13* clades.

Recently, several MADS-domain-containing genes have been isolated, their expression patterns studied and in some instances in vivo functions deciphered through reverse genetic approaches (Reichmann and Meyerowitz 1997; Schmidt and Ambrose 1998). We have attempted to obtain a hint of *OsMADS1* function by examining its evolutionary relationship with other genes, particularly those of the *AP1/AGL9* gene clades. The related evolutionary histories of individual gene groups could reflect their distinct functional specificity in regulating floral cell fate. The amino acids corresponding to the full length predicted OsMADS1 protein align well with predicted proteins for most members of this group particularly in the following domains: MADS box, intermediate linker

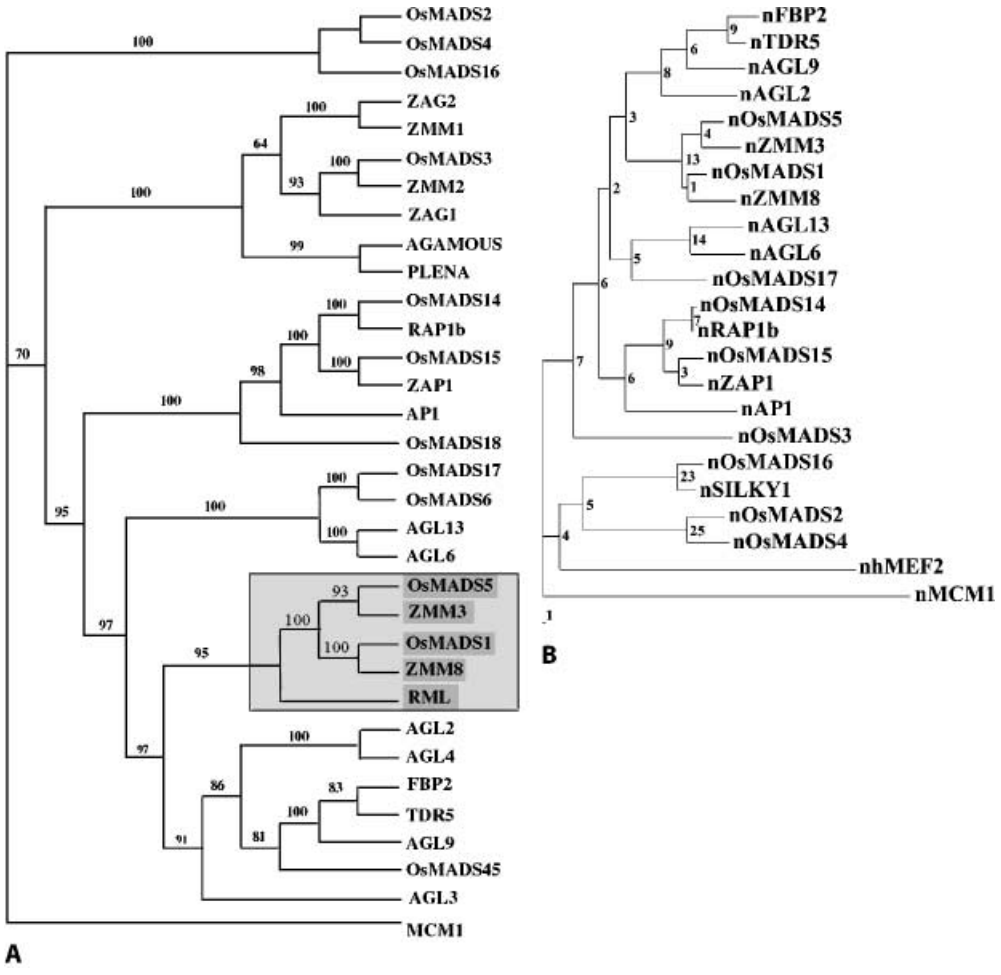

Fig. 1 **A** Phylogenetic tree for the rice *OsMADS1*, *OsMADS5* and *AP1*, *AGL9*-like MADS-box genes from other genera. A selected number of *AG*-related genes were also used in the analysis. Maximum parsimony based analyses was performed for the predicted full-length protein sequences. The yeast MCM1 protein was used as the outgroup. *Numbers* (written above each node) give the Bootstrap values from 100 replicates. Nodes with <50% bootstrap support are collapsed. The tree length is 2,408 steps. The topology of the tree remained the same even when the Heuristic search algorithm was employed. The *shaded box* indicates the *OsMADS1* clade. **B** Neighbor-Joining-based tree showing evolutionary distances for plant MADS-box-containing genes. The *scale bar* indicates the number of nucleotide substitutions. The distance matrix was determined for nucleotide sequences corresponding to the MADS-intermediate-keratin-like (MIK) domains of the indicated proteins, by determining the Tajima-Nei nucleotide substitution rate

region and the K box. These regions comprise the DNA binding domain, connecting linker or intermediate domain and the predicted coiled-coil domain with structural similarity to the fibrous protein keratin, respectively. The alignment was unambiguous for protein as well as the nucleotide sequences that code for the MIK region of the proteins (data not shown). The phylogenetic relatedness of *OsMADS1* to members of the *AP1* and *AGL9* clades from *Arabidopsis*, *Petunia*, tomato, rice and maize was examined. In addition, relationship to members of the *AG* clade from *Arabidopsis*, *Antirrhinum*, rice and maize was also determined. We employed the maximum parsimony method (MP) and phylogenetic trees were generated by NJ to determine its position within this gene family. A single tree was derived from parsimony analysis with high bootstrap support in 100 replicates [consistency index CI=0.6952, retention index =0.6732, rescaled consistency index (RC)=0.4680]. This analysis included the yeast MCM1 protein as the outgroup (Fig. 1A). The tree thus generated was largely similar to one where the *Arabidopsis* AGAMOUS protein was used as the outgroup (data not shown). Interestingly, while *OsMADS1* belongs to the *AP1/AGL9* lineage, it clearly forms a distinct subgroup with the maize genes *ZMM8* and *ZMM3*. Furthermore, *OsMADS1* and *ZMM8* are the two rice and maize genes that are most closely related in sequence (80.97% overall identity and 100% identity in MADS box). In addition, *OsMADS5*, a closely related rice gene, also belongs to this sub-group. Notably, this sub-group is distinct from the previously designated orphan group of genes typified by *Arabidopsis AGL6* and *AGL13* genes. The *OsMADS1* sub-group seems to be unique to the monocots with no sequence homologues as yet from any dicot species, despite the near complete sequence information from the *Arabidopsis* genome. Phylogenetic relationships using distance-based criteria were also determined and this was performed for nucleotide sequences corresponding to the MIK region of the derived proteins. The data was again used to generate a tree with NJ where bootstrap values were used to indicate the reliability of the branches (see Materials and methods). This analysis deduced a lineage similar to that obtained from analysis of the predicted protein (Fig. 1B). Thus, both analyses place *OsMADS1* and its likely maize homologues in a monophyletic sub-group that has diverged from the *AGL9* clade. The topology of the tree derived with the distance matrix correlates well with the cladogram derived using protein sequences for the entire gene.

Different domains of plant MADS-box genes show differing rates of non-synonymous substitutions, with the

Table 1 Estimates of non-synonymous nucleotide substitution rates within MADS, I and K domains of pairs of genes. *OsMADS1* is compared to *AGL9*, or *AG*-related factors (*MIK* MADS intermediate keratin-like domain)

	MADS	K-box	MIK
OsMADS5	0.020	0.095	0.091
ZMM8	0.000	0.036	0.050
ZMM3	0.015	0.079	0.077
TDR5	0.091	0.353	0.290
ZAP1	0.080	0.458	0.361
RAP1b	0.091	0.392	0.328
AP1	0.136	0.499	0.401
AGL9	0.060	0.337	0.278
AGL13	0.079	0.510	0.370
OsMADS3	0.123	0.737	0.459

lowest degree of change occurring in the MADS box which is constrained by its DNA binding property. Comparing the levels of non-synonymous substitutions can provide clues on functional divergence of specific domains for members of this family. We have compared the proportion of non-synonymous substitution in the gene pairs *OsMADS1* versus *ZMM8*, *OsMADS5*, *ZMM3*, *AP1* or *AGL9*. In this analysis we have taken the maize *ZAP1*, rice *RAP1b* and *OsMADS3*, tomato *TDR5* and *Arabidopsis AGL13* as divergent sequences (Table 1). Despite accounting for the constraints on the MADS box, these analyses suggest *OsMADS1* to be distinct from other molecules of the *AP1* or *AGL9* clade and its divergence possibly reflects its differing functional specificity. Significantly, all of these analyses place this sub-group as one that is different from that of *AGL13* and *AGL6*. Collectively, these data hint at a unique function for *OsMADS1* and *OsMADS5* in rice panicle and flower development.

Expression profile of *OsMADS1* during panicle development

AP1, *AGL9* and its likely orthologues from *Antirrhinum*, *Petunia* and tomato are the best characterized among the genes in this group. *AP1* is a floral meristem identity gene and the earliest amongst the MADS-box genes to be expressed exclusively in floral tissues. Its temporally early expression in very young floral primordia is uniform and precedes the onset of expression of the other floral organ identity genes. The temporally later expression of *AP1* is only in the perianth structures: whorls 1 and 2 (Mandel et al. 1992b). This spatial restriction occurs concomitant to the expression of the organ identity MADS-box gene *AG* in presumptive whorls 3 and 4 (Gustafson-Brown et al. 1994). In contrast, *AGL9* as well as its putative *Petunia* and tomato orthologues *FBP2* and *TM5*, are expressed uniformly in the young floral meristem. However, the onset of *AGL9* expression is after that of *AP1*. In more mature flowers undergoing organogenesis the *AGL9* group of genes are expressed in three inner whorls: petals, stamens and carpels (Angenent et al. 1992; Pneuli et al.1994; Mandel and Yanofsky 1997). Other members of this clade are expressed in all four whorls of the flower. Not surprisingly, the rice genes such as *OsMADS24* and *OsMADS45* that are closely related to *AGL9* (Fig. 1A) share similarities in expression profiles with these dicot genes. *OsMADS45* and *OsMADS24* are expressed in lodicules, stamens and carpel of rice flowers (Greco et al. 1997).

Initial studies on *OsMADS1* expression revealed high levels of expression in the flower primordium, a feature similar to that of *AP1*- and *AGL9*-like factors (Chung et al. 1994; Vijayraghavan 1996). The developmentally later expression pattern of *OsMADS1* differs in that it is expressed strongly in lemma, palea and weakly in the carpel. The lemma and palea are modified glumes, suggested by certain criteria to be analogous to sepals of dicot flowers (Schmidt and Ambrose 1998). The rice flower bears additional pair of glumes peripheral to the lemma and palea. We have examined whether *OsMADS1* is a likely determinant of lemma/palea cell fate by examining in detail its expression pattern in the developing panicle, particularly in very early flower primordia where the outer glumes are established, but the lemma and palea are yet to be formed. We observe that *OsMADS1* RNA is first detected in the incipient floral primordium where it is excluded from outer sterile glumes that subtend the flower primordia (Fig. 2A, B, and C, D). *OsMADS1* is not expressed in earlier stages of panicle development, i.e. during primary or secondary rachis branch formation (Fig. 2A, B). Its earliest expression occurs well after differentiation of the panicle branches. Therefore, *OsMADS1* and *RAP1A* are currently the earliest markers to be expressed exclusively in the rice floral meristem **(**Fig. 2 and Kyozuka et al. 2000). As reported earlier, the early uniform expression of *OsMADS1* in the wild-type floral meristem (Fig. 2C, D) is later confined to strong expression in the lemma and palea primordia at about the stage of their initiation (Fig. 2E, F). *OsMADS1* RNA is completely excluded from the lodicule and stamen primordia and spikelets where organ differentiation is occurring (Fig. 2E–J). Notably, weak but clearly detectable levels of *OsMADS1* RNA are seen in the carpel primordia (Fig. 2E, F, and G, H). In contrast, *RAP1A* is expressed in the lemma, palea and lodicules, with low levels of RNA in the sterile glumes (Kyozuka et al. 2000). These differences in expression patterns reinforce the differences between these two genes hinted at by our phylogenetic analysis (Fig. 1). Prior suggestions of distinctions between the lemma (bract) and palea (prophyll; reviewed in Schmidt and Ambrose 1998) are not reflected in the expression pattern of *OsMADS1* which is indistinguishable in these two structures. In this aspect, *OsMADS1* and *RAP1A* are similar. The developmentally late expression pattern of *OsMADS1* in the lemma, palea and carpels of rice flowers varies from the known pattern of expression for *AGL9* group of MADS-domain proteins, including those known in rice.

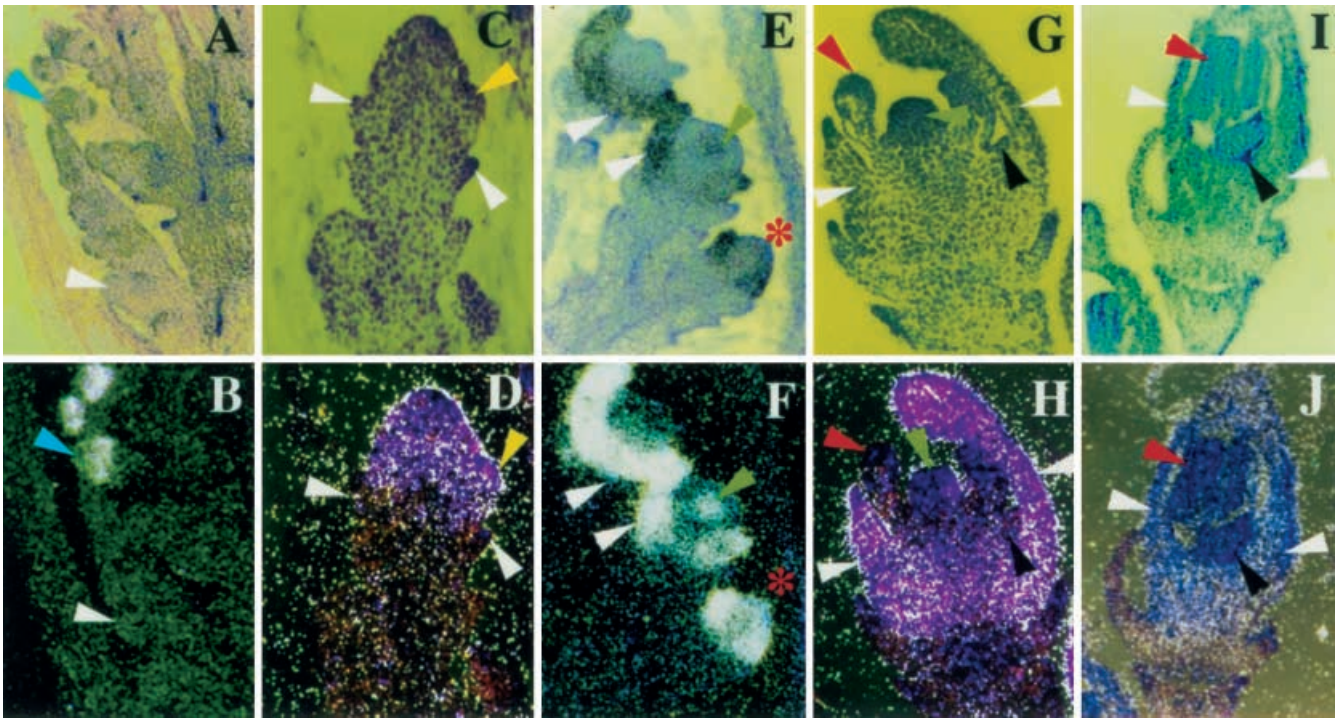

Fig. 2A–J The RNA expression pattern of *OsMADS1* in secondary rachis branch primordia (*srb*), very young flower primordia, and flower primordia with differentiating organs. In situ hybridization of longitudinal sections of wild-type tissues with an *OsMADS1*-specific mRNA probe is shown**. A**, **C**, **E**, **G, I** Bright-field photographs; the corresponding dark field images are **B**, **D**, **F**, **H** and **J**. RNA expression was detected as the *silver grains* in the dark field images. **A, B** Young panicle with developing srb (*white arrowhead*) and young spikelet primordia (*blue arrowhead*). **C, D** Spikelet primordium at the stage of lemma specification; outer rudimentary glumes (*white arrowheads*) and lemma primordium (*yellow arrowhead*). **E, F** Spikelets during organ primordia specification; young spikelet primordium (*red star*) slightly later in development than that in **C**, lemma/palea (*white arrowhead*), and carpel primordium in an older spikelet (*green arrowhead*). **G, H** Spikelet with differentiating organ primordia; lemma and palea (*white arrowhead*), lodicule (*black arrowhead*), stamen (*red arrowhead*), and carpel (*green arrowhead*). **I, J** Spikelet undergoing anther differentiation; lemma and palea (*white arrowhead*), lodicule (*black* arrowhead), and anther lobe (*red arrowhead*)

Ectopic expression of *OsMADS1* affects the panicle morphology

In wild-type plants (TP309), the inflorescence stem develops as a central rachis bearing 6–8 nodes. Each of these nodes produces primary rachis branches (prb) that in turn bear 6–9 nodes. Basal nodes of the prb bear secondary rachis branches (srb), while apical nodes bear individual spikelet primordia. A spikelet primordia in rice bears a single bisexual flower, and these primordia are also generated on the srb (Hoshikawa 1989). A single spikelet in rice contains a pair of sterile glumes that remain small and underdeveloped (Fig. 4A) compared to that observed in other cereal flowers, for example, sorghum, wheat or oat. Internal to these glumes, the rice spikelet bears a single flower with a lemma, a palea, two

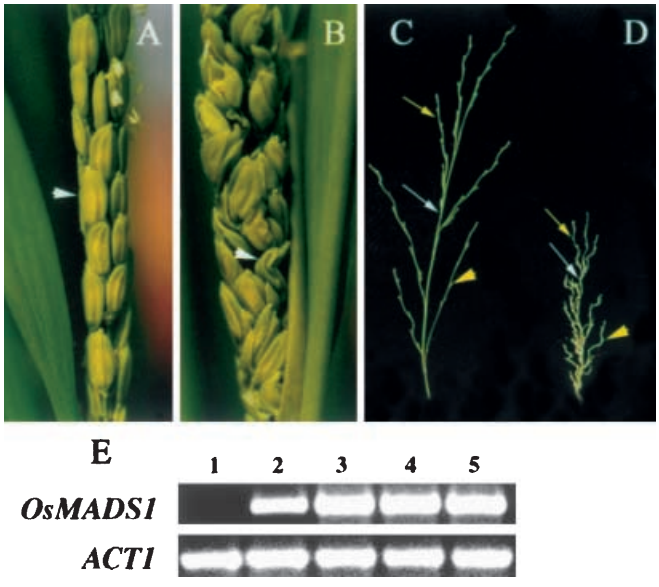

Fig. 3 Morphology of panicles from wild-type plants (**A**) versus pUBI-*OsMADS1* transgenic plants (**B**) at the stage of panicle heading for the wild-type plant. The stunted panicle in the T0 transgenic plant remained embedded in leaf sheath. The disorganized positioning of the spikelets gives a severely twisted phenotype (*white arrowhead* in **B**). **C** A fully emerged wild-type panicle where seeds/spikelets are removed to display the rachis branches. *White arrow* central rachis, *yellow arrow* primary rachis branch, *yellow arrowhead* secondary rachis branch. **D** T0 *OsMADS1* transgenic panicle with spikelets removed to show the severely shortened central rachis and branches thereof. **E** RT-PCR analysis of *OsMADS1* transcripts in transgenic lines. Total RNA from leaves of transgenic plants were used in RT-PCR with primers for either the 541-bp C-terminal fragment of *OsMADS1* (*upper panel*) or the 588-bp fragment of the constitutively expressed endogenous rice *ACT1* (*lower panel*). *Lane 1* RT-PCR product from vector pCAMBIA 1381Xc-transformed plants. *Lanes 2–5* RT-PCR products from four independent Ubi-*OsMADS1* lines

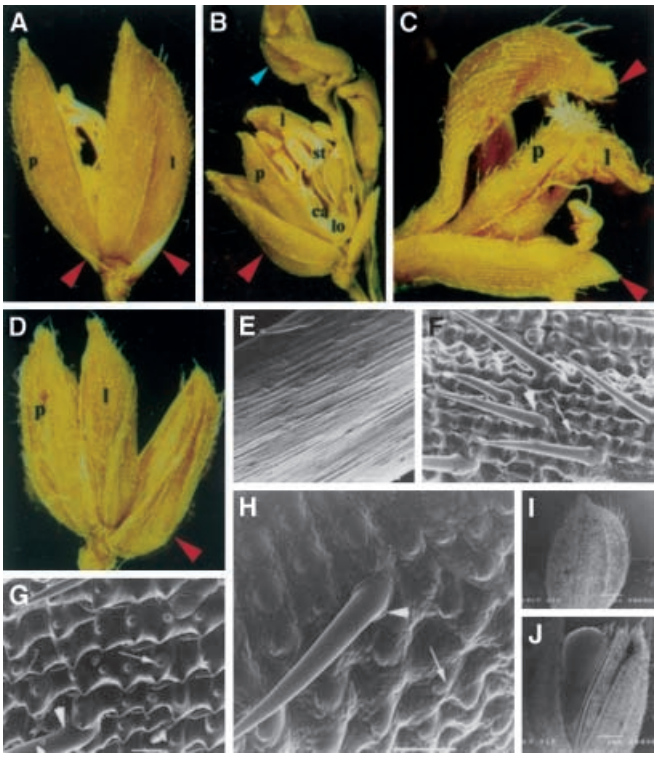

Fig. 4A–J Homeotic phenotypes of *OsMADS1* transgenic spikelets. **A** Wild-type spikelet with rudimentary outer glumes (*red arrowheads*), lemma (*l*) and palea (*p*). **B** A portion of a T0 transgenic panicle, with one spikelet partially dissected open; the outer glume (*red arrowhead*) is converted into a lemma/palea-like organ. The other rudimentary glume, while enlarged, is smaller than the first one. Inner to these glumes are the lemma, palea, lodicule (*lo*), stamens (*st*) and carpel (*ca*). A deformed spikelet with premature termination of differentiation is shown on the same panicle branch (*blue arrowhead*). **C** Spikelet where both the glumes (*arrowheads*) are enlarged and transformed into lemma/palea-like organs. **D** Spikelet with one outer glume, converted into a lemma/palea-like structure. Internal to this are found the normal lemma and palea, but there are no further internal organs. **E**–**G** Scanning electron micrographs (SEM) of the outer surface of rudimentary glume (**E**), lemma (**F**), and palea (**G**) of a wild-type spikelet. **H** SEM of the epidermal cell surface of a transformed outer glume in a T0 spikelet. *White arrows* in **F**, **G**, and **H** point to several epidermal cells with protrusions typical of lemma/palea and *white arrowheads* indicate trichomes. **I** SEM of a wild-type seed enclosed by the lemma and palea. **J** Transgenic seed partially enclosed by the lemma/palea and transformed glumes. *Scale bars* 50 µM in **E**–**H** and 1 mm in **I**, **J**

lodicules, six stamens and a central carpel. For the sake of convenience, we have used the term rice flower primordium and rice spikelet primordium interchangeably here. We have used ectopic expression studies as a tool to examine the functional contribution of *OsMADS1* to rice panicle and spikelet formation.

An *OsMADS1* cDNA clone was isolated from the rice panicle cDNA library and then sub-cloned under maize ubiquitin promoter to generate pUbi-*OsMADS1* construct (see Materials and methods). This construct was transformed by *Agrobacterium*-mediated transformation into embryogenic rice calli. Plants from four independent hygromycin-resistant calli were regenerated. During vegetative development, plants transgenic for *OsMADS1* did not differ from control vector transformed plants. The parameters of plant height, leaf development and number of tillers per plant (branches from basal vegetative nodes) remained similar to those in controls. Panicle heading was also comparable. A reduction in flowering time by a few days and overall dwarfing upon *OsMADS1* expression was recently reported by Jeon et al. (2000). The differences between our observations may lie in the photoperiods during transgenic plant growth. This possibility can be tested to examine if *OsMADS1* contributes to flowering time in a photoperiod-sensitive pathway.

All the plants from four independent Ubi-*OsMADS1* lines show defects in panicle development. The panicles are shorter in length, severely defective for internode elongation, and have abnormally positioned branch primordia or lateral primordia on the main axis (Fig. 3C vs D). The panicle length is reduced to about 60% of that in control plants (Fig. 3C vs D, and Table 2). This is one of the two general features noted in the recent study of Jeon et al. (2000). We detail here our further characterization of the panicle defects. Significant reduction in length of prb, which are in average only 65% of the length of control branches, was observed (Table 2). Additionally, a striking finding is that the prb are also highly deformed in shape with lateral primordia (i.e. srb primordia and flower primordia) arising at irregular and atypical intervals (Fig. 3D). While the panicle rachis branches are atypical in these plants the number of nodes remains the same as in control panicles (Table 2). The disorganized

Table 2 Panicle characteristics in control vector-transformed plants (*1381Xc*) or pUbi-*OsMADS1*-transformed plants (*prb* primary rachis branches)

Transgene	Length of panicle in cm±SD [a]	Number of nodes on central rachis±SD	Length of prb in cm±SD	Number of nodes on prb±SD	Number of spikelets on each panicle±SD
1381Xc no. 1	11.5±1.3	6.0±0.8	5.6±0.4	8.0±0.5	68.0±5.9
1381Xc no. 2	10.9±1.0	6.7±0.7	4.7±0.6	7.2±0.4	64.0±6.0
1381Xc no. 3	12.1±1.0	6.2±0.8	6.0±0.6	7.6±0.4	62.0±5.8
1381Xc no. 4	11.3±0.9	6.4±0.8	6.3±1.0	7.0±0.5	71.0±5.6
pUbi-OsMADS1 no. 1	5.9±0.9	6.0±0.7	2.1±0.5	6.0±0.7	61.2±5.2
pUbi-OsMADS1 no. 2	4.7±1.0	5.6±0.6	2.7±0.5	7.2±0.8	59.1±6.0
pUbi-OsMADS1 no. 3	5.2±0.9	6.6±0.7	2.4±0.4	5.9±0.6	64.2±5.8
pUbi-OsMADS1 no. 4	4.9±0.8	5.9±0.8	2.6±0.4	6.7±0.8	66.2±5.1

[a] Panicle length is represented as length of central rachis. At least seven panicles of each line were examined

arrangement of the flowers on the shortened panicle and rachis branches contributes to its compact appearance (Fig. 3B). Spikelet morphology was also affected; these data are discussed in the following section. To establish that the panicle phenotype results from ectopic expression of *OsMADS1* and not co-suppression (silencing) of the endogenous *OsMADS1* gene, we have determined the steady state levels of *OsMADS1* RNA in vegetative tissues of these transgenic lines. The ubiquitin promoter is expected to direct constitutive expression of *OsMADS1* RNA in these transgenic lines. RT-PCR was carried out to measure *OsMADS1* RNA levels as compared to those of the endogenous rice actin mRNA. We found a high level and stable expression of *OsMADS1* RNA that was quantitatively comparable to that of actin (*ACT1*) mRNA (Fig. 3E). Together, these results suggest that ectopic expression of *OsMADS1* is responsible for the stunted transgenic panicles with their distinct phenotypes. Recently, it has been suggested that floral homeotic genes differentially regulate the cell division patterns during various stages of *Arabidopsis* flower development (Jenik and Irish 2000). Possibly, the ectopically expressed *OsMADS1* exerts its effects on the early stage of panicle branching by altering patterns of cell proliferation.

OsMADS1 ectopic expression results in homeotic transformation of outer glumes into lemma/palea-like organ

As mentioned earlier, rice spikelet has a pair of rudimentary outer glumes at its base, inner to which are the larger, well developed modified glumes: the lemma and palea. These latter organs enclose the internal three whorls of floral organs. By the criteria of *OsMADS1* gene expression pattern, and also that of rice *RAP1A* or the maize *ZAP1*, it is inferred that lemma and palea define floral organs analogous to first whorl sepals of other flowers (Chung et al. 1994; Mena et al. 1995; Vijayraghavan 1996; Ambrose et al. 2000; Kyozuka et al. 2000). A different line of support for this hypothesis comes from phenotypic analysis of a maize mutant: *Silky1*, where lodicules are replaced by lemma/ palea structures; a transformation consistent with a class B loss-of-function mutation (Ambrose et al. 2000). The reasonable prediction is that in grass flowers orthologues of the dicot ABC floral organ identity genes pattern the lemma/palea, lodicules, stamens and carpels. Since the *OsMADS1* gene, by sequence relatedness and by expression pattern differs from the above-mentioned molecules, we anticipated that the consequences of its ectopic expression on spikelet structure would be informative vis-a-vis its function.

A consistent phenotype in a significant proportion (~42%) of flowers in all independently generated *OsMADS1* transgenic lines was a drastic enlargement of one pair of the outer glumes. The normally rudimentary glumes immediately peripheral to the lemma and palea now approach the size of the lemma or the palea (Fig. 4C). The normal lemma and palea are found inner to these transformed glumes. A weaker phenotype observed was flowers wherein only one glume was transformed to a lemma/palea like structure; the other glume remained at its normal size. Internal to the transformed glume was the lemma and palea, yet many of such flowers did not bear any stamen or carpel (Fig. 4D). However, a small proportion of the flowers displayed transformation of one glume to a lemma/palea like structure, with otherwise normal floral organ patterning (Fig. 4B). Thus, the most notable phenotype was the presence of over-developed outer glumes, a characteristic also seen by Jeon et al. (2000). We have examined further if these over-developed glumes are merely the result of increase in glume size, or whether they originate from homeotic transformation of a pair of normally rudimentary glumes to lemma/palea. We have determined the epidermal cell surface characteristics of the rudimentary glume, lemma and palea in wild-type flowers and then used their distinguishing features to determine the organ and cellular identities in the over-developed glumes of transgenic flowers. The rudimentary glume bears epidermal cells organized as long files of smooth cells with few trichomes present only on the edges (Fig. 4E). The lemma is typified by epidermal cells with rounded projections and also by a high density of trichomes (Fig. 4F). The wild-type palea bears similar cell morphology as the lemma but has fewer trichomes (Fig. 4G). The epidermal cells of the transformed glume of transgenic flowers has characteristics of both lemma and palea, i.e. cells with rounded epidermal projections and interspersed trichomes (Fig. 4H). These features prove the homeotic transformation of glumes to lemma/palea. Thus *OsMADS1* expression in rudimentary outer glumes is sufficient for their conversion into lemma-palea like organs. Possibly, *OsMADS1* achieves this function by targeting downstream genes needed for differentiation of lemma and palea cell types. A speculative function for the *Arabidopsis AGL9* like genes is that they act as cofactors for B and C floral organ identity genes (Pelaz et al. 2000). The closely related rice genes *OsMADS24* and *OsMADS45* (Fig. 1) could function similarly and contribute to lodicule, stamen and carpel differentiation in rice. In a similar manner, a plausible hypothesis for *OsMADS1* action in the sterile glumes is as a partner/cofactor for gene/s expressed in these glumes. *RAP1A*, which is expressed at low levels in outer sterile glumes, could be one such candidate partner. In this scenario, ectopic expression of *OsMADS1* possibly facilitates the transformation of glumes to lemma/palea like organs. However, we do not observe any transformation of the lodicule in transgenic Ubi-*OsMADS1* plants. Thus, it is probable that in lodicules, interaction of *RAP1A* with other homeotic genes precludes the effects of *OsMADS1* ectopic expression therein. Studying the consequences of loss-of-function and gain-of-function of *RAP1A* in Ubi-*OsMADS1* transgenic lines can test these hypotheses.

Since the total number of the flowers in these panicles is similar to that in control panicles (Table 2) we conclude that the flowers which do not bear any internal or-

gans are not supernumerary flowers generated as a consequence of ectopic expression of *OsMADS1*. Instead, the data suggest that ectopic expression promotes the precocious assignment of floral meristems on the branches, perhaps at the expense of branch primordia differentiation. Additionally, because there is a sizeable fraction of flowers with underdeveloped floral organs, particularly those with no carpels, we hypothesize that increased expression of *OsMADS1* (gain-of-function) promotes increased determinacy of fourth whorl cells. This speculation is supported by observations on phenotypic consequence of *OsMADS1* loss-of-function. The *lhs* loss-of-function mutant arises from mutations in the MADS box of *OsMADS1*, and the consequences are under-developed leafy lemma and palea, leafy lodicules, decreased stamens and occasional additional carpel or flower (Jeon et al. 2000). However these mutant flowers have normal rudimentary outer glumes as in wild type. Unlike *lhs* loss-of-function mutant, Ubi-*OsMADS1* spikelets show homeotic conversion of outer sterile glumes into lemma -palea like organs without affecting the lodicule morphology. These contrasting phenotypes are consistent with a role for *OsMADS1* and possibly a partner gene expressed in outer glumes in assigning lemma/palea fate. In strong loss-of-function *lhs* alleles, internal flowers are generated within a spikelet. These observations suggest a loss-of determinacy in the flower primordium in these mutants. These data, together with our observations of underdeveloped carpels in pUbi-*OsMADS1* spikelets, implies that *OsMADS1* plays an important role in conferring determinacy to the floral meristem center i.e., the fourth whorl. They also suggest that *OsMADS1* possibly functions as a floral meristem as well as organ identity gene, though the mechanism by which it defines the floral meristem remains to be examined.

The closely related *OsMADS5* gene, whose product shares 72% identity with *OsMADS1*, perhaps shares some functions with *OsMADS1* (Kang and An 1997). Preliminary studies on the consequences of its ectopic expression in a heterologous system: tobacco, showed weak early flowering (Kang and An 1997). No data was gathered on floral organ types. The individual and combined contributions of *OsMADS1* and *OsMADS5* can be examined in rice plants that bear loss-of-function/gain-of-function alleles for both loci. The genes *ZMM8/ZMM3* could be the orthologues in maize for the *OsMADS1/OsMADS5* genes, and may similarly be involved in functions distinct from that of previously described *AP1* or *AGL9* family of genes. Since no *Arabidopsis* gene that is closely related to this sub-group has been found as yet, the role of such factors in dicot flowers remains to be investigated.

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