

Identification of a rice *APETALA3* homologue by yeast two-hybrid screening

Yong-Hwan Moon, Ji-Young Jung, Hong-Gyu Kang and Gynheung An*

Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

(*author for correspondence)

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Abstract

A cDNA clone *OsMADS16* was isolated from the rice young inflorescence cDNA expression library by the yeast two-hybrid screening method with *OsMADS4* as bait. We have previously shown that the *OsMADS4* gene is a member of the *PI* family and that the MADS-box gene is involved in controlling development of the second and third whorls of rice flowers. The sequence comparison indicated that *OsMADS16* belongs to the *AP3* family. The *OsMADS16* protein contains a PI-derived motif, FAFRVVPSQPNLH, that is a conserved sequence in *AP3* family genes at the C-terminal region. In addition, *OsMADS16* contains a paleoAP3 motif, YGGNHDLRLG, downstream of the PI-derived motif. The paleoAP3 motif is a consensus sequence in the C-terminal region of the *AP3* family genes of lower eudicot and magnolid dicot species. RNA blot analysis showed that the *OsMADS16* gene was expressed in the second and third whorls, whereas the *OsMADS4* transcripts were present in the second, third, and fourth whorls. These expression patterns of the *OsMADS16* and *OsMADS4* genes are very similar to those of *AP3* and *PI*, respectively. In the yeast two-hybrid system, *OsMADS4* interacted only with *OsMADS16* among several rice MADS genes investigated, suggesting that *OsMADS4* and *OsMADS16* function as a heterodimer in specifying sepal and petal identities. The *OsMADS16* protein displayed transcription activation ability in yeast, whereas *AP3* did not. It was also shown in yeast that *OsMADS16* interacted with *PI* whereas *OsMADS4* did not interact with *AP3*. These differences between *OsMADS16* and *AP3* indicate that the functions of the *AP3* family genes of monocots and dicots diverged during molecular evolution processes of the B function genes. Deletion analysis showed that the 155–200 amino acid region of the *OsMADS16* protein plays an important role in the transcription activation ability.

Introduction

Recent studies on flower development of various dicot species including *Arabidopsis thaliana*, *Antirrhinum majus*, petunia, and tomato have elucidated that the genetic and molecular mechanisms controlling flower organ identity have been highly conserved (Coen, 1991; Weigel and Meyrowitz, 1994). It has been established that three classes of homeotic genes, A, B, and C, which encode proteins containing a highly con-

served MADS-box motif, specify the identities of the floral organs in dicots. The class A genes in whorl 1 and the class C genes in whorl 4 function to develop sepals and carpels, respectively. In combination, the class A and B genes determine the fate of petals in whorl 2. Similarly, the class B and C genes together control formation of stamens in whorl 3. The class A MADS genes include *APETALA1* (*API*) in *Arabidopsis* (Mandel *et al.*, 1992; Gustafson-Brown *et al.*, 1994) and *SQUAMOSA* (*SQUA*) in *Antirrhinum* (Huijser *et al.*, 1992), the class B genes include *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* (Jack *et al.*, 1992; Goto and Meyerowitz, 1994)

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF077760.

and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum* (Sommer *et al.*, 1990; Trobner *et al.*, 1992), and the class C genes include *AGAMOUS* (*AG*) in *Arabidopsis* (Yanofsky *et al.*, 1990) and *PLENA* (*PLE*) in *Antirrhinum* (Bradley *et al.*, 1993). In addition to specification of stamen and carpel identity, the class C genes inhibit the indeterminate growth of the floral meristem (Mizukami and Ma, 1992, 1995).

In dicot plants, the class B MADS genes are required to specify petal and stamen identities. Ectopic expression of both *PI* and *AP3* in *Arabidopsis* plants resulted in the transformation of sepals into petals and carpels into stamens, demonstrating that the activities of these two genes together are sufficient to provide the B function (Krizek and Meyerowitz, 1996). Also, simultaneous expression of both *DEF* and *GLO* in tobacco plants caused extreme alterations of the floral organs (Davies *et al.*, 1996b). The conversion of the first whorl sepals to petals was almost complete, accompanying pigment development and morphological alteration. These ectopic expression analyses indicate that the functions of the B group genes appear to be limited to establishing organ identity.

In vitro DNA-binding studies showed that the *AP3* and *PI* proteins specifically bind, as a heterodimer, to the CARG motif found in the promoter region of *AP3* and other floral homeotic genes (Riechmann *et al.*, 1996a,b; Hill *et al.*, 1998; Tilly *et al.*, 1998). It was reported that heterodimerization between *AP3* and *PI* was necessary for nuclear localization of these proteins (McGonigle *et al.*, 1996). *In vitro* binding experiments suggested that the MADS box, the entire I region, and the first putative amphipathic helix of the K box region of the *AP3* and *PI* genes are responsible for the heterodimerization (Riechmann *et al.*, 1996b). In addition, it was demonstrated that the MADS box and I region are involved in nuclear localization (McGonigle *et al.*, 1996). It was shown that the K-box region, a domain found only in plant MADS-box factors, plays an important role in protein-protein interaction (Davies *et al.*, 1996a). These observations suggest that the B-class genes function only as a heterodimer during the processes of DNA binding, nuclear localization, and protein-protein interaction.

Although the B-class genes of *Arabidopsis* and *Antirrhinum* have many similarities in their function (Irish and Yamamoto, 1995), a closer look at the putative class B orthologous genes *AP3* and *DEF* shows that they differ in the expression patterns, the phenotypes of fourth-whorl organs in their mutants, and transcriptional dependence on their B class partner

genes (Schwarz-Sommer *et al.*, 1990; Jack *et al.*, 1992; Goto and Meyerowitz, 1994; Jack *et al.*, 1994; Samach *et al.*, 1997). Furthermore, the *AP3* family genes of higher eudicot, lower eudicot, and magnolid dicot species have a different conserved motif in the C-terminal region; higher eudicots have an euAP3 motif, D(L/I)TTFALLE, whereas lower eudicots and magnolid dicots have a paleoAP3 motif, YGxHDLRLA (Kramer *et al.*, 1998). These differences in the conserved motifs of the *AP3* family genes indicate that the *AP3* lineage has experienced a significant diversification.

Although much is known about the function of class B genes of dicot plants, those of monocot plants have been reported only in a few species. In rice, we have previously isolated two *PI* family genes, *OsMADS2* and *OsMADS4* (Chung *et al.*, 1995). They were expressed in young inflorescences, stamens, and carpels, showing that the expression patterns of the MADS genes are similar to those of *PI*. Transgenic rice plants expressing antisense *OsMADS4* displayed alterations of the second and third whorls (Kang *et al.*, 1998). The second-whorl lodicules were altered into palea/lemma-like organs, and the third-whorl stamens were changed to carpel-like organs. This result suggests that *OsMADS4* belongs to the *PI* gene family.

The yeast two-hybrid system provides a sensitive and specific method to study protein-protein interactions. It was previously shown that *DEF* and *GLO* specifically select each other in the system, demonstrating usefulness of the method (Davies *et al.*, 1996a). In this study, we have isolated an *AP3* family gene of rice, *OsMADS16*, by the yeast two-hybrid system using *OsMADS4* as bait.

Materials and methods

Plasmid construction

The binding domain vector, pBDGAL4, and the activation domain vector, pADGAL4, were purchased from Stratagene. The sequences containing K and C regions (KC), K domain (K) or C-region (C) in *OsMADS1*, *OsMADS3*, *OsMADS4*, *OsMADS5*, *OsMADS6*, *OsMADS7*, *OsMADS8*, *OsMADS16*, *AP3*, and *PI* were generated by polymerase chain reaction (PCR). For all constructs, the 5' *EcoRI* site and the 3' *SalI* site were introduced by PCR. PCR was performed to amplify the following regions: *OsMADS1*-KC, amino acid (aa) residues 85–257; *OsMADS3*–

KC, 87–236; OsMADS4-KC, 84–210; OsMADS5-KC, 89–225; OsMADS6-KC, 86–250; OsMADS7-KC, 90–249; OsMADS8-KC, 90–248; OsMADS16-KC, 83–223; OsMADS16-K, 83–173; OsMADS16-C_{155–180}, 155–180; OsMADS16-C_{155–200}, 155–200; OsMADS16-C_{155–223}, 155–223; OsMADS16-C_{181–223}, 181–223; OsMADS16-C_{201–223}, 201–223; AP3-KC, 83–232; PI-KC, 83–208. The AP3-KC and PI-KC clones were isolated from the total cDNA of *Arabidopsis* flower buds. The PCR profile used was 1 min at 95 °C, 1 min at 57 °C, and 1.5 min at 72 °C for a total of 40 cycles. The PCR products were digested with *EcoRI* and *SalI*, ligated to pBDGAL4 or pADGAL4, and their sequences were determined to confirm the proper fusion of the constructs.

Construction of expression cDNA library

Total RNA was isolated from rice young inflorescence when the length of the panicles was below 2 cm using the RNA isolation kit (TRI Reagent, Molecular Research Center). mRNA was isolated using the Poly(A) Quick mRNA isolation kit (Stratagene, La Jolla, CA). The expression cDNA library was made by the standard method according to the HybriZAP two-hybrid cDNA Gigapack cloning kit manual (Stratagene) using Uni-ZAP XR and HybriZAP vectors as cloning vectors. The initial pfu of the Uni-ZAP cDNA library and the HybriZAP cDNA library were 5.85×10^6 and 4.94×10^6 , respectively. Total cDNA of the phagemid form was obtained by the mass *in vivo* excision method.

Yeast two-hybrid screening

The yeast strain YRG-2 (*Mata*, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *gal4-542*, *gal80-538*, *LYS::UAS_{GAL1}-TATA_{GAL1}-HIS3*, *URA3::UAS_{GAL4}^{17mers(x3)}-TATA_{CYC1}-lacZ*), was purchased from Stratagene. YRG-2 was transformed with pBD/OsMADS4-KC, the binding domain plasmid containing the K domain and C region of OsMADS4, according to the modified procedure of the lithium acetate method (Gietz *et al.*, 1992). The transformants were tested for *HIS3* reporter gene expression and found not to express the *HIS3* gene as indicated by no growth on a medium lacking histidine. The strain was used for transformation using 100 µg plasmid DNA of the HybriZAP cDNA library and 3 mg of salmon sperm carrier DNA by the lithium acetate method. All of the transformation mixture was

plated on the synthetic dropout (SD) medium (SD-Trp-Leu-His + 3-AT) lacking tryptophan, leucine, and histidine, and containing 1 mM 3-aminotriazole (3-AT) (Kaiser *et al.*, 1994). About 1.2×10^6 transformants were obtained as estimated based on the number of transformants on the SD-Trp-Leu plate. Thirty colonies that grew on the SD-Trp-Leu-His + 3-AT plates after five days were transferred subsequently onto the filter on the SD-Trp-Leu-His plate and incubated for one day. The β -galactosidase activity was measured by the filter assay according to Breeden and Nasmyth (1985). The colonies that turned blue in less than 6 h were selected. DNA from the selected colonies was rescued and retransformed into YRG-2 with pBDGAL4 or pBD/OsMADS4-KC. Plasmid DNA was recovered from yeast colonies according to Hoffman and Winston (1987), and transformed into an *E. coli* strain, XL1-blue, by electroporation.

Isolation of the 5' region of the OsMADS16 cDNA

The 5' region of the OsMADS16 cDNA was isolated by PCR with the T3 primer (5'-AATTAACCCTCACTAAAGGG-3') and two OsMADS16-specific primers (388–407, 5'-GTCCAGATCTTCTCCCATCC-3'; and 548–568, 5'-GCTCCTGCTGCAGAGTCTTG-3'). The template was a total cDNA, which was *in vivo* excised from the Uni-ZAP cDNA library. The amplified fragment was cloned into pBSII KS(+) (Stratagene). Double-stranded DNA was used as a template for DNA sequence analysis following the manufacturer's instructions (Amersham, Thermo Sequase cycle sequencing kit). Amino acid sequence homology comparison was performed with the BLASTX alignment (Altschul *et al.*, 1997).

DNA blot analysis

Genomic DNA was prepared from rice leaves according to the protocol of Shure *et al.* (1983). A 10 µg portion of DNA was digested with *EcoRI*, *HindIII*, or *PstI*, subjected to electrophoresis in a 0.8% agarose gel, and then blotted onto a Hybond-N+ filter (Amersham). The K box fragment (273 bp from 312 to 584) of the *OsMADS16* cDNA clone was labeled by the random priming method (Amersham) and used as a probe. The filter was prehybridized for 3 h at 65 °C in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS (Church and Gilbert, 1984). Hybridization was performed for 20 h at 65 °C in a prehybridization solution supplemented with labeled probes. The filter was

A

1 MGRGKIEIKRIKRNATNROVTYSKRRTGIMKKARELTVLCDAQVALIMESS
51 TGKYHEEFCSPSTDIKGIFDRYQQAIGTSLWIEQYENMQRTLSHLKDINRN
101 LRTEIRQRMGEDLDGLEFDELRLGLEQNVDAALKEVRRHRKYHVISTQTETY
151 KKKVKHSYEAYKTLQQELGLCEEPAWFVDNTGGGWDGGAGAGAAADMFAP
201 RVVPSQPNLHGMAYGGNHDLRLA*

B

AADMFAFRVVPVPS---QPN--LHGM-A--YGGNHDLRLA	223	OsMADS16
GPNI F AFRLQPS---QPN--LHN--GGG-YNCHDLRLA	228	PnAP3-2
VHNL Y AFRLQPLH---PN--LQNE--GG--FGSRDLRLS	222	TM6
		YGxHDLRLA
		PaleoAP3 Motif
GSRAYALRFHQNH HHYYPNHGLH APSAS--DITTFHLE	232	AP3
----LRFHQNH HHYYPNHGLH APSAS--DITTFHLE	224	BobAP3
GPRIT AL RLPTNHH--PT--LH--SGGSDLTTFALLE	227	DEF
		D(L/I)ITTFALLE
		EuAP3 Motif
FxFRLQPSQPNLH		PI Motif-derived
S-MPFF FRVQPSHPNLQ QEK---	210	OsMADS4
-QVPF GFQVFPMPQPNL TTVT TTTT TNNK	231	PnPI-1
-Q--FGYRVQPIQPNLQEKIMSLVID	208	PI
-QMPFA FRVQPMQPNLQ ERF---	215	GLO
MPE x FRVQPMQPNLQ		PI-Motif

Figure 1. A. The deduced amino acid sequence of *OsMADS16*. MADS box region is underlined and K box region is indicated in bold. B. Alignment of amino acid sequences of C-terminal region of *OsMADS16* and another B class MADS genes. *PnAP3-2*, *TM6*, *AP3*, *BobAP3*, and *DEF* are members of the *AP3* family genes of *Papaver nudicaule*, tomato, *Arabidopsis thaliana*, *Brassica oleracea*, and *Antirrhinum majus*, respectively. The *OsMADS4*, *PnPI-1*, *PI*, and *GLO* are members of the *PI* family genes of rice, *Papaver nudicaule*, *Arabidopsis thaliana*, and *Antirrhinum majus*, respectively. A gray-shaded background denotes amino acid sequences of paleoAP3 motif, euAP3 motif, PI motif-derived motif, or PI motif.

washed three times for 5 min in 0.2× SSPE and 0.1% SDS at 65 °C.

RNA blot analysis

Total RNA was isolated by the RNA isolation kit (TRI Reagent, Molecular Research Center) from leaves, roots, lodicules, sterile lemma, palea lemma, stamens, and carpels. A 20 μg portion of total RNA was fractionated on a 1.3% agarose gel as described previously (Sambrook *et al.*, 1989). After RNA transfer onto a nylon membrane, the blots were prehybridized, hybridized, and washed with the same method used in the DNA blot analysis except for temperature which was 60 °C. The K-box fragments of the *OsMADS4* (231 bp

between 369 and 599) and *OsMADS16* cDNA clones were labeled and used as probes.

Quantitative assay of β-galactosidase activity

Mid-to-late exponential-phase yeast cells were collected and resuspended in a Z buffer (Miller, 1972). The cells were assayed for β-galactosidase activity as described by Miller (1972) using *O*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate. The activity unit was calculated using the formula: 1000× OD₄₂₀/(OD₆₀₀ × assay time in min × assay volume in ml).

Table 1. Quantitative assay of protein-protein interaction between OsMADS4 and rice MADS proteins.

Activation domain plasmid	β -Galactosidase activity ^a	
	OsMADS4-KC ^b	pBDGAL4 ^c
OsMADS1-KC	0.15 ± 0.048	0.04 ± 0.011
OsMADS3-KC	0.14 ± 0.039	0.04 ± 0.013
OsMADS4-KC	0.15 ± 0.045	0.04 ± 0.013
OsMADS5-KC	0.16 ± 0.046	0.05 ± 0.014
OsMADS6-KC	0.15 ± 0.033	0.03 ± 0.012
OsMADS7-KC	0.15 ± 0.045	0.04 ± 0.013
OsMADS8-KC	0.17 ± 0.031	0.04 ± 0.011
OsMADS16-KC	59.70 ± 3.860	0.03 ± 0.010
None	0.12 ± 0.042	0.04 ± 0.013

^a β -galactosidase activity unit = $1000 \times \text{OD}_{420} / [\text{OD}_{600} \times \text{reaction time (min)} \times \text{volume of culture (ml)}]$.

^bGAL4 DNA binding domain fusion protein.

^cpBDGAL4 is used as a negative control.

Results

Two-hybrid screening

To identify proteins that interact with OsMADS4, a truncated OsMADS4 protein containing the K domain and C region was fused to the GAL4 DNA-binding domain. The resulting plasmid pBD/OsMADS4-KC was introduced into the yeast strain YRG-2 and the transformants were tested for activation of the *HIS3* selectable marker. The transformants did not grow on a medium lacking histidine, demonstrating that OsMADS4 does not contain an activator domain in the K domain and C region. We, therefore, proceeded to introduce the cDNA expression library constructed from the mRNA of rice young flowers. A total of 1.2×10^6 transformants were screened for their ability to grow on a medium lacking histidine. This initial screening identified 30 colonies, which subsequently were tested for activation of the *lacZ* gene. These experiments resulted in identification of seven colonies. Plasmid DNAs were prepared from these colonies and retransformed into the YRG-2 strain in order to confirm whether the activation is indeed due to the presence of the fusion protein. We observed that all of the seven plasmids were able to activate the *lacZ* gene only in the presence of pBD/OsMADS4-KC. Sequence determination of the seven clones revealed that, although they had different length inserts, they all encoded an identical protein. The deduced amino acid sequences of the clones were highly homologous to MADS proteins in the AP3 family.

Isolation of the cDNA clone containing an entire open reading frame

The clones selected by the yeast two-hybrid screening were partial, lacking the 5' region that encodes the N-terminal end of the protein. The 5' region was isolated by PCR using a vector primer and two cDNA-specific primers. A cDNA clone of 1059 bp containing the entire open reading frame was generated by connecting the 5' region to the cDNA clone obtained from the yeast two-hybrid screening. It contains the 65 bp 5'-untranslated region, 300 bp 3'-untranslated region, and an open reading frame of 223 amino acid residues (calculated molecular mass 25.4 kDa). The gene corresponding to this clone was designated *OsMADS16*. The OsMADS16 protein shows 56.6% identity with PnAP3-2, an AP3 orthologue of *Papaver nudicaule* (Kramer *et al.*, 1998), 49.8% with DEF (Sommer *et al.*, 1990), and 49.3% with AP3 (Jack *et al.*, 1992). The OsMADS16 protein contains the MADS box domain which consists of 56 conserved amino acids present in the N-terminal region of all of the MADS transcription factors (Figure 1). The K-box domain, a region considered to participate in protein-protein interaction, is also present in between amino acid residues 89 and 154. In the C-terminal region, two consensus amino acid sequences are located (Figure 1). One is the PI motif, Fx~~F~~R~~L~~Q~~P~~S~~Q~~P~~N~~L~~H~~, which is found in most of the AP3 family genes. The other is the paleoAP3 motif, YGxHDLRLA, which is a conserved sequence in the AP3 family MADS genes of lower eudicot and magnolid dicot species (Kramer *et al.*, 1998).

Expression patterns of OsMADS4 and OsMADS16

It was well established that there are a large number of MADS genes in the rice genome (Chung *et al.*, 1994, 1995; Kang *et al.*, 1995, 1997; Kang and An, 1997). Therefore, it was necessary to identify the region of the *OsMADS16* cDNA that does not hybridize with other genes in order to study expression patterns of the genes. Genomic DNA blot analysis showed that one prominent band was specifically hybridized with the 273 bp fragment between nucleotides 312 and 584, which is located at the K region (Figure 2).

RNA blot analyses were conducted using this gene-specific probe and RNAs isolated from leaves, roots, and various floral organs. In rice, the spikelet, the unit of inflorescence, consists of a lemma, a palea, two lodicules, six stamens, and a carpel. The

Table 2. Protein-protein interaction assay between the B class proteins of rice and *Arabidopsis*.

Binding domain plasmid	Activation domain plasmid	β -Galactosidase activity ^a
AP3-KC	PI-KC	27.42 \pm 1.957
PI-KC	AP3-KC	30.40 \pm 0.463
AP3-KC	AP3-KC	0.11 \pm 0.032
PI-KC	PI-KC	0.13 \pm 0.024
OsMADS16-K	OsMADS4-KC	80.67 \pm 5.630
OsMADS16-K	PI-KC	53.26 \pm 4.342
PI-KC	OsMADS16-KC	69.58 \pm 5.520
OsMADS16-K	AP3-KC	0.11 \pm 0.014
AP3-KC	OsMADS16-KC	0.07 \pm 0.022
OsMADS4-KC	AP3-KC	0.12 \pm 0.012
AP3-KC	OsMADS4-KC	0.10 \pm 0.027
OsMADS4-KC	PI-KC	0.09 \pm 0.021
PI-KC	OsMADS4-KC	0.07 \pm 0.039
AP3-KC	–	0.09 \pm 0.012
PI-KC	–	0.11 \pm 0.022
OsMADS16-K	–	0.09 \pm 0.032
OsMADS4-KC	–	0.12 \pm 0.042
–	AP3-KC	0.05 \pm 0.012
–	PI-KC	0.07 \pm 0.013
–	OsMADS16-KC	0.08 \pm 0.011
–	OsMADS4-KC	0.08 \pm 0.013

^a β -galactosidase activity unit = $1000 \times OD_{420} / [OD_{600} \times \text{reaction time (min)} \times \text{volume of culture (ml)}]$.

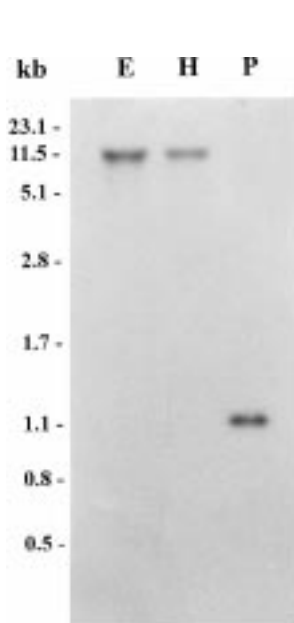


Figure 2. Southern blot analysis of *OsMADS16*. The rice genomic DNA was digested with *EcoRI* (E), *HindIII* (H), and *PstI* (P), respectively. The numbers indicate the size, in kb, of DNA markers.

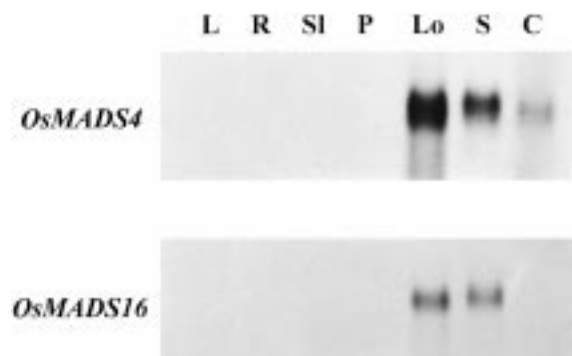


Figure 3. RNA blot analysis of *OsMADS4* and *OsMADS16*. Ethidium bromide staining of 25S and 17S rRNAs demonstrated equal amounts of RNA loading (data not shown). L, leaves; R, roots; Sl, sterile lemma; P, palea/lemma; Lo, lodicules; S, stamens; C, carpels.

OsMADS16 transcript was present in lodicules and stamens, but not detectable in other organs (Figure 3). Lodules are small, oval, and fleshy organs located immediately outside of the stamens and are considered to be petal (Yoshida, 1981). We have previously shown that the *OsMADS4* gene is expressed specifically in stamens and carpels (Chung *et al.*, 1998). However,

the expression pattern in lodicules and sterile lemmas had not been investigated. In this study, it was observed that the *OsMADS4* transcript is present in lodicules, stamens, and carpels; the lodicules showed the strongest expression and the carpels showed the weakest level of expression (Figure 3). These expression patterns of the *OsMADS4* gene and *OsMADS16* gene are similar to those of the *PI* gene and *AP3* gene in *Arabidopsis*, respectively; the *PI* transcript is present in the second, third, and fourth whorls, and the *AP3* transcript in the second and third whorls (Jack *et al.*, 1992; Goto and Meyerowitz, 1994).

Interaction between OsMADS4 and other MADS box proteins

The yeast two-hybrid screening resulted in identification of only one type of MADS gene when *OsMADS4* was used as a bait. In order to determine whether *OsMADS4* specifically interacts with *OsMADS16* as a heterodimer, we investigated protein-protein interactions between *OsMADS4* and other rice MADS proteins. The C-terminal half containing the K domain and C region of *OsMADS1*, 3, 4, 5, 6, 7, 8, and 16 was fused with the activation domain vector pADGAL4. The plasmid pBD/*OsMADS4*-KC was used as a bait. The colonies that grew on a medium lacking leucine and tryptophan were examined for β -galactosidase activity (Table 1). These analyses showed that *OsMADS4* interacts only with *OsMADS16* among the examined rice MADS genes, suggesting that *OsMADS4* and *OsMADS16* function as a heterodimer in specifying sepal and petal identities.

Cross-interaction ability between the B-class MADS genes

The sequence identity and expression patterns of the class B genes of rice and *Arabidopsis* suggested that the genes were highly conserved during evolution. If their functional roles have been preserved, they should interact with their counterparts of heterologous origin. The yeast two-hybrid system was used to study interactions between the B class proteins. As a control, interaction between *AP3* and *PI* was examined. When the K and C regions of the *AP3* and the *PI* proteins were fused to pBDGAL4 or pADGAL4, these fusion proteins of *AP3* and the *PI* interacted with each other to activate the *lacZ* reporter gene (Table 2). On the other hand, *AP3* and *PI* did not interact with themselves. These results showed that the yeast two-hybrid system reproduced the property

of the *AP3* and *PI* proteins, which specifically bind each other to form a heterodimer. When pBD/*PI*-KC and pAD/*OsMADS16*-KC or pBD/*OsMADS16*-K and pAD/*PI*-KC were introduced into YRG-2, the *lacZ* gene was activated, demonstrating that *OsMADS16* interacts with *PI*. This experiment showed that the dimerization site of the *OsMADS16* protein is equivalent to that of *AP3*. In contrast to *OsMADS16*, the dimerization site of the *OsMADS4* protein was not equivalent to that of *PI*. The yeast two-hybrid system demonstrated that *OsMADS4* did not interact with *AP3* or *PI* (Table 2).

Identification of transcription activation ability of OsMADS4, OsMADS16, AP3, and PI

The transcription activation ability of the B class MADS genes from rice as well as those from *Arabidopsis* was investigated using the yeast two-hybrid system. The C-terminal half of the MADS proteins containing the K domain and C region was connected to the GAL4 DNA binding domain. The resulting constructions, pBD/*OsMADS4*-KC, pBD/*OsMADS16*-KC, pBD/*AP3*-KC, and pBD/*PI*-KC plasmids were introduced into the yeast strain YRG-2. The transformants that grew on a medium lacking tryptophan were examined for activation of the *lacZ* gene by the X-gal filter assay and β -galactosidase activity analysis (Table 3). Interestingly, only the pBD/*OsMADS16*-KC plasmid activated the *lacZ* gene, suggesting that the C-terminal half of *OsMADS16* has transcription activation ability.

Identification of transcription activation motifs in OsMADS16

In order to narrow down the region that is involved in the transcription activation ability of the *OsMADS4* protein, the K domain and C region were separated and each half was connected to the GAL4 binding domain and tested in yeast. While the K domain did not activate the *lacZ* gene, the C region between amino acid residues 155 and 223 contained the transcription activation ability (Table 3). However, the C-terminal region carrying the residues between 181 and 223 did not show any transcription activation ability. Likewise, the residues between 201 and 223 had no activity, indicating that the sequence between 155 and 180 is essential for the activation. Gain-of-function experiments revealed that the 26 aa residues between 155 and 180 were not sufficient, but that the 46 aa residues

between 155 and 200 carried the transcription activation ability. These results suggest that the region between amino acid residues 155 and 200 plays an important role in transcription activation ability of the OsMADS4 protein.

Discussion

We have isolated the *OsMADS16* gene by yeast two-hybrid screening using OsMADS4 as a bait. The protein is most homologous to various MADS genes of the AP3 family. In the C-terminal region, OsMADS16 contained the sequence FAFRVVPSQPNLH, which is a conserved motif in the AP3 family, supporting that *OsMADS16* is a member of the AP3 family. Interestingly, OsMADS16 contains the sequence YG-GNHDLRLG, which is the paleoAP3 motif found in the AP3 family of lower eudicot and magnolid dicot species, such as *Medicago sativa*, *Pachysandra terminalis*, *Papaver californicum*, *Papaver nudicaule*, *Dicentra eximia*, *Caltha palustris*, *Michelia figo*, and *Peperomia hirta* (Heard and Dunn, 1995; Kramer *et al.*, 1998). Although tomato belongs to the higher eudicot family, *TM6*, an AP3 family gene of tomato, has the paleoAP3 motif (Pnueli *et al.*, 1991). The AP3 family genes of most of higher eudicot species such as *Antirrhinum majus*, *Arabidopsis thaliana*, *Petunia hybrida*, *Solanum tuberosum*, *Nicotiana tabacum*, *Brassica oleracea*, and *Silene latifolia* contain the euAP3 motif, D(L/I)TTFALLE, in the C-terminal region (Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1992; Garcia-Maroto *et al.*, 1993; van der Krol *et al.*, 1993; Hardenack *et al.*, 1994; Davies *et al.*, 1996b; Carr and Irish, 1997). The maize AP3 homologue *SILKY-1* contains the paleoAP3 motif with one amino acid difference (C. Padilla and R. Schmidt, personal communication). The sequence properties of maize and rice suggest that the AP3 family genes of monocots are more closely related to the lower eudicot species than those of higher eudicot species.

In mature floral organs, *OsMADS16* was expressed in lodicules and stamens, whereas *OsMADS4* was in lodicules, stamens, and carpels. These organ-specific expression patterns of *OsMADS4* and *OsMADS16* are identical to those of *PI* and *AP3*, respectively, indicating functional similarity between these MADS genes (Jack *et al.*, 1992; Goto and Meyerowitz, 1994).

We investigated the protein-protein interaction between OsMADS4 and rice MADS genes using the yeast two-hybrid system. Various rice MADS genes,

Table 3. Assay of the transcription activation ability of OsMADS4, OsMADS16, PI, and AP3.

Binding domain plasmid	β -Galactosidase activity ^a
AP3-KC	0.09 \pm 0.012
PI-KC	0.11 \pm 0.022
OsMADS4-KC	0.12 \pm 0.042
OsMADS16-KC	72.98 \pm 7.188
OsMADS16-K	0.09 \pm 0.032
OsMADS16-C ₁₅₅₋₂₂₃	65.12 \pm 4.070
OsMADS16-C ₁₈₁₋₂₂₃	0.12 \pm 0.023
OsMADS16-C ₂₀₁₋₂₂₃	0.07 \pm 0.039
OsMADS16-C ₁₅₅₋₁₈₀	0.25 \pm 0.008
OsMADS16-C ₁₅₅₋₂₀₀	145.61 \pm 7.673

^a β -galactosidase activity unit = 1000 \times OD₄₂₀/[OD₆₀₀ \times reaction time (min) \times volume of culture (ml)].

OsMADS1, 3, 4, 5, 6, 7, 8, and 16, were selected as interaction partners of OsMADS4. Based on the sequence homology analysis and the antisense approach, it was previously determined that *OsMADS1* (Chung *et al.*, 1994), *OsMADS5* (Kang and An, 1997), *OsMADS6* (our unpublished results), *OsMADS7*, and *OsMADS8* (Kang *et al.*, 1997) are members of the AGL2 family, *OsMADS3* is a member of the AGAMOUS family (Kang *et al.*, 1995), and *OsMADS4* is a member of the PI family (Chung *et al.*, 1995; Kang *et al.*, 1998). The result revealed that OsMADS4 interacted only with OsMADS16. This specific interaction between B function genes as a heterodimer has been reported in the class B genes of dicots such as *Arabidopsis* and *Antirrhinum* (Davies *et al.*, 1996a; McGonigle *et al.*, 1996; Riechmann *et al.*, 1996a,b). Yeast two-hybrid screening with DEF as bait identified only GLO as a partner and vice versa (Davies *et al.*, 1996a). In this protein-protein interaction between DEF and GLO, the K domain played an important role. We observed the same result that the K domain plays an important role in interaction between OsMADS4 and OsMADS16 (data not shown). The importance of the K domain in protein-protein interaction between MADS proteins was also reported in yeast two-hybrid experiments with AG as a bait (Fan *et al.*, 1997). The K domain of AG was able to bind to the K domains of AGL2, AGL4, AGL6, and AGL9.

The interaction studies between the rice and *Arabidopsis* B function proteins revealed that OsMADS16 cross-interacted with PI, but OsMADS4 did not interact with AP3 (Table 2). The result shows that while OsMADS16 is able to replace AP3, OsMADS4 cannot substitute PI, indicating the diversity of the B class

genes during molecular evolution in monocots and dicots. It was proposed that the *AP3* and *PI* family genes are the products of a duplication event (Doyle, 1994; Purugganan *et al.*, 1995; Theissen *et al.*, 1996; Purugganan, 1997). According to this model, the euAP3 lineage containing *PI* and *AP3* was evolved from the paleoAP3 lineage by duplication events. While the *PI* family appears to be highly conserved, the *AP3* family has experienced significant sequence diversification (Kramer *et al.*, 1998). The *AP3* family genes also exhibit functional diversification in several aspects. First, the *Arabidopsis AP3* is expressed in petals and stamens whereas the *Antirrhinum* orthologue, *DEF*, is expressed in carpels as well as in petals and stamens (Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1992). Second, while mutations in *DEF* eliminated fourth-whorl organs, *AP3* mutations did not affect fourth-whorl organ development (Jack *et al.*, 1992, 1994; Schwarz-Sommer *et al.*, 1992). Finally, the *DEF* gene expression depends on a functional GLO protein, whereas transcriptional regulation of the *AP3* gene seems to be independent of the PI protein (Schwarz-Sommer *et al.*, 1992; Goto and Meyerowitz, 1994).

While the OsMADS16 protein displayed transcription activation ability in yeast, OsMADS4, AP3, and PI did not show any activation ability (Table 3). This transcription activation ability of OsMADS16 was limited to the C-terminal region since the K region alone did not activate the *lacZ* gene in a yeast two-hybrid system. Deletion analysis in the C-terminal region showed that the region between amino acid residues 155 and 200 plays an essential role. This region, however, did not contain an acidic domain, a glutamine-rich region, or a proline-rich region, which are characteristics of transcription activators (Hope and Struhl, 1986; Ma and Ptashne, 1987; Courey *et al.*, 1989; Mermod *et al.*, 1989; Aeschbacher *et al.*, 1991; Schindler *et al.*, 1992). Whether OsMADS16 indeed functions as a transcription activator in floral organ development remains to be resolved in a plant system.

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