

Determination of the Motif Responsible for Interaction between the Rice APETALA1/AGAMOUS-LIKE9 Family Proteins Using a Yeast Two-Hybrid System¹

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A MADS family gene, *OsMADS6*, was isolated from a rice (*Oryza sativa* L.) young flower cDNA library using *OsAMDS1* as a probe. With this clone, various MADS box genes that encode for protein-to-protein interaction partners of the *OsMADS6* protein were isolated by the yeast two-hybrid screening method. On the basis of sequence homology, *OsMADS6* and the selected partners can be classified in the *APETALA1/AGAMOUS-LIKE9* (*AP1/AGL9*) family. One of the interaction partners, *OsMADS14*, was selected for further study. Both genes began expression at early stages of flower development, and their expression was extended into the later stages. In mature flowers the *OsMADS6* transcript was detectable in lodicules and also weakly in sterile lemmas and carpels, whereas the *OsMADS14* transcript was detectable in sterile lemmas, paleas/lemmas, stamens, and carpels. Using the yeast two-hybrid system, we demonstrated that the region containing of the 109th to 137th amino acid residues of *OsMADS6* is indispensable in the interaction with *OsMADS14*. Site-directed mutation analysis revealed that the four periodical leucine residues within the region are essential for this interaction. Furthermore, it was shown that the 14 amino acid residues located immediately downstream of the K domain enhance the interaction, and that the two leucine residues within this region play an important role in that enhancement.

The vegetative-to-reproductive transition is an important event in a plant's development. Upon initiation of the reproductive phase, the development of the floral meristem is initiated by floral meristem identity genes such as *LFY* (*LEAFY*) and *AP1* (*APETALA1*) in Arabidopsis (Mandel et al., 1992; Weigel et al., 1992; Hempel et al., 1997). At a later step, the fate of floral organ primordia is specified by three classes of homeotic genes (for review, see Weigel and Meyerowitz, 1994): *AP1*, *AG* (*AGAMOUS*), *PI* (*PISTILATA*), and *AP3* in Arabidopsis (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994), and *SQUA* (*SQUAMOSOSA*), *PLE* (*PLENA*), *GLO* (*GLOBOSA*), and *DEF* (*DEFICIENS*) in snapdragon (Sommer et al., 1990; Huijser et al., 1992; Tröbner et al., 1992; Bradley et al., 1993). These homeotic genes belong to the MADS-domain protein gene family. MADS box genes encode a family of highly conserved transcription factors that participate in signal

transduction and developmental control in plants, animals, yeast, and fungi.

In addition to the three classes of organ identity genes in plants, there are a large number of other MADS box genes whose function is less well defined. In Arabidopsis at least 17 *AGL* (*AG-LIKE*) genes have been isolated (Ma et al., 1991; Mandel and Yanofsky, 1995; Rounsley et al., 1995). It was revealed that some of these genes are floral organ specific and appear to be involved in controlling floral organ initiation and development. For example, *AGL2* and *AGL4* are first expressed very early in flower development (after the floral meristem has emerged from the inflorescence meristem but before any of the organ primordia emerge), suggesting that *AGL2* and *AGL4* play important roles in the intermediate step between inflorescence initiation and floral organ initiation (Flanagan and Ma, 1994; Savidge et al., 1995). In addition to flower development, several MADS box genes are involved in the control of ovule and seed development, vegetative growth, root development, embryogenesis, or symbiotic induction (Mandel et al., 1994; Angenent et al., 1995; Heard and Dunn, 1995; Flanagan et al., 1996; Perry et al., 1996; Buchner and Boutin, 1998; Zhang and Forde, 1998).

Plant MADS box proteins consist of a MADS box domain, an I region, a K domain, and a C-terminal region. The conserved MADS box domain is required for sequence-specific DNA binding and dimerization (Mizukami et al., 1996; Riechmann et al., 1996a, 1996b; West et al., 1998). The MADS box domains bind to the consensus DNA sequence, the CarG motif (Huang et al., 1993, 1995; Tilly et al., 1998). In *AP3* and *PI* the MADS box and the I region are needed for nuclear localization of the proteins (McGonigle et al., 1996). The K domain is the second conserved region, carrying 65 to 70 amino acid residues and located in the middle of the MADS box proteins. The K domain was named due to its structural resemblance to the coiled-coil domain of keratin, and has been suggested to be involved in protein-to-protein interactions (Ma et al., 1991; Pnueli et al., 1991; Theissen et al., 1995). The C-terminal region is rich in acidic amino acids, which are characteristic of transactivation domains. Using the yeast two-hybrid system, we recently demonstrated that *OsMADS16*, a rice (*Oryza sativa* L.) *AP3* homolog, contains a transcription activation domain in the C-terminal region of the protein (Moon et al., 1999).

It has been demonstrated that the K domain is required for interactions between MADS box proteins (Davies et al., 1996; Fan et al., 1997). In experiments using the yeast

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two-hybrid system, GLO and DEF, the B class proteins of snapdragon, specifically selected each other as a partner in the protein-to-protein interaction, and the K domain played an important role in the interaction (Davies et al., 1996). Furthermore, in studies using the same system, AG interacted with AGL2, AGL4, AGL6, and AGL9 (Fan et al., 1997). In that study it was demonstrated that the K domain is necessary in the protein-to-protein interaction. These results indicate that MADS box proteins cooperate with other MADS box proteins in a K-domain-mediated interaction to carry out their functions.

Almost all of our knowledge about the interaction between MADS box proteins has been obtained from the two dicots Arabidopsis and snapdragon. Although many MADS box proteins were isolated from monocots, including maize, sorghum, orchid, and rice (Lu et al., 1993; Schmidt et al., 1993; Chung et al., 1995; Kang et al., 1995, 1997; Mena et al., 1995; Montag et al., 1995; Greco et al., 1997; Kang and An, 1997), the interaction between the MADS box proteins has not been elucidated. We previously reported the isolation of *OsMADS1*, a rice MADS box gene that exhibited the highest homology with *AGL2* (Chung et al., 1994). It was demonstrated that ectopic expression of the *OsMADS1* gene in homologous and heterologous plants resulted in early flowering, suggesting that the rice *MADS* gene is involved in flower induction (Chung et al., 1994). In the present study, we report the isolation of a rice *API/AGL9* family gene, *OsMADS6*, by screening a young flower cDNA library from rice using *OsMADS1* as a probe. With this gene we isolated the protein-to-protein interaction partners by the yeast two-hybrid system. We also investigated the motif responsible for the interaction between MADS box proteins.

MATERIALS AND METHODS

Library Screening and Sequence Analysis

The expression cDNA libraries were constructed from mRNA isolated from young rice (*Oryza sativa* L.) panicles (less than 2 cm height) using Uni-ZAP XR and HybriZAP vectors (Stratagene; Moon et al., 1999). Total cDNAs of a phagemid form were obtained by the mass in vivo excision method. Hybridization was performed with 10^5 plaques using a labeled probe prepared from the *OsMADS1* coding region. The cDNA clones were rescued by in vivo excision using a helper phage (ExAssist, Stratagene). Double-stranded DNA was used as a template for DNA sequence analysis following the manufacturer's instructions (Thermo Sequase cycle sequencing kit, Amersham). Amino acid sequence homology was compared using the BLASTX alignment program (Altschul et al., 1997).

Plasmid Construction

The binding domain vector pBDGAL4 and the activation domain vector pADGAL4 were purchased from Stratagene. The sequences containing a portion of *OsMADS1*, *OsMADS3*, *OsMADS4*, *OsMADS5*, *OsMADS6*, *OsMADS7*, *OsMADS8*, *OsMADS14*, *OsMADS15*, *OsMADS17*, and *Os-*

MADS18 were generated by PCR. DNA sequences encoding the following amino acid residues were amplified: 85 to 257 in *OsMADS1* (*OsMADS1-KC*), 87 to 236 in *OsMADS3* (*OsMADS3-KC*), 84 to 210 in *OsMADS4* (*OsMADS4-KC*), 89 to 225 in *OsMADS5* (*OsMADS5-KC*), 1 to 90 in *OsMADS6* (*OsMADS6-MI*), 1 to 170 in *OsMADS6* (*OsMADS6-MIKC14*), 86 to 156 in *OsMADS6* (*OsMADS6-K*), 86 to 170 in *OsMADS6* (*OsMADS6-KC14*), 86 to 250 in *OsMADS6* (*OsMADS6-KC*), 90 to 249 in *OsMADS7* (*OsMADS7-KC*), 90 to 248 in *OsMADS8* (*OsMADS8-KC*), 1 to 90 in *OsMADS14* (*OsMADS14-MI*), 1 to 172 in *OsMADS14* (*OsMADS14-MIKC14*), 90 to 172 in *OsMADS14* (*OsMADS14-KC14*), 90 to 246 in *OsMADS14* (*OsMADS14-KC*), and the K domain and C region of *OsMADS15* (*OsMADS15-KC*), *OsMADS17* (*OsMADS17-KC*), and *OsMADS18* (*OsMADS18-KC*). Regions containing amino acids 86 to 110 (*OsMADS6-KI*), 109 to 137 (*OsMADS6-KII*), 138 to 170 (*OsMADS6-KIII*), and 109 to 170 (*OsMADS6-KII+KIII*) of *OsMADS6* were also amplified by PCR.

Replacement mutants in the K box region of *OsMADS6* were generated by PCR as follows: Leu-110 with Ser and Leu-118 with Arg in the KII fragment (*OsMADS6-KIIS₁₁₀R₁₁₈*); Leu-126 with Arg and Leu-134 with Arg in the KII fragment (*OsMADS6-KIIR₁₂₆R₁₃₄*); Leu-110 with Ser and Leu-118 with Arg in the KII and KIII fragments (*OsMADS6-KIIS₁₁₀R₁₁₈+KIII*); Leu-159 with Arg and Leu-166 with Arg in the KII and KIII fragments (*OsMADS6-KII+KIIR₁₅₉R₁₆₆*); and Leu-110 with Ser, Leu-118 with Arg, Leu-159 with Arg, and Leu-166 with Arg of the KII and KIII fragments (*OsMADS6-KIIS₁₁₀R₁₁₈+KIIR₁₅₉R₁₆₆*). For all constructs, the 5' *EcoRI* site and the 3' *Sall* site were introduced by PCR. 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 *Sall*, ligated to pBDGAL4 or pADGAL4, and transformed into appropriate hosts. The sequences of all inserts were determined to confirm the proper fusion of the constructs.

Yeast Two-Hybrid Screening

The yeast (*Saccharomyces cerevisiae*) strain YRG-2 (*Mata α* , *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/*OsMADS6-KC14*, the binding domain plasmid containing the K domain and 14 amino acid residues of the C-terminal region of *OsMADS6*, using a modified lithium acetate method (Gietz et al., 1992). The transformants were tested for imidazoleglycerol-phosphate dehydratase (*HIS3*) reporter gene expression and found not to express this gene, as indicated by the absence of growth on a medium lacking His.

The strain was transformed with 100 μ g of plasmid DNA of the HybriZAP (Stratagene) cDNA library, along with 3 mg of salmon-sperm carrier DNA, using the lithium acetate method. The transformants were plated on SD (synthetic dropout) medium lacking Trp, Leu, and His, and containing 1 mM 3-aminotriazole (SD-Trp-Leu-His+3-AT; Kaiser et al., 1994). Approximately 1.4×10^6 transformants were

obtained, as estimated based on the number of transformants grown on the SD-Trp-Leu plate. The 59 colonies that grew on the SD-Trp-Leu-His+3-AT plates after 5 d were subsequently transferred onto the filter paper on the SD-Trp-Leu-His plate and incubated for 1 d. The β -galactosidase activity was measured by filter assay according to the method of Breeden and Nasmyth (1985). The colonies that turned blue in less than 6 h were selected for isolation of DNA, and were then retransferred into the YRG-2 yeast strain containing pBDGAL4 or pBD/OsMADS6-KC14. Plasmid DNA was recovered from yeast according to the method of Hoffman and Winston (1987) and transformed into *Escherichia coli* strain XL-1-Blue (Stratagene) by electroporation.

Isolation of the 5' Region of the OsMADS14 cDNA

The 5' region of the OsMADS14 cDNA was isolated by PCR using the T3 primer (5'-AATTAACCTCACTA-AAGGG-3') and the *OsMADS14* gene-specific primer (5'-ATGGACTCGAGCATTAGTTGG-3') located within the K domain. The template was total cDNA in vivo excised from the Uni-ZAP (Stratagene) cDNA library. The amplified fragment was cloned into pBlueScript II KS(+) (Stratagene).

DNA and RNA Blot Analyses

Genomic DNA was prepared from 2-week-old rice seedlings according to the protocol of Shure et al. (1983). DNAs (10 μ g) were digested with *EcoRI*, *HindIII*, or *PstI*, subjected to electrophoresis in a 0.8% (w/v) agarose gel, and then blotted onto a Hybond-N⁺ filter (Amersham). The filter was prehybridized, hybridized, and washed according to the method of Moon et al. (1999).

Total RNA was isolated (TRI reagent, Molecular Research Center, Cincinnati) from young flowers with a panicle size of 1 to 5 cm, flowers at the early vacuolated pollen stage, flowers at the late vacuolated pollen stage, leaves, roots, lodicules, sterile lemmas, paleas/lemmas, stamens, and carpels. Total RNA (20 μ g) was fractionated on a 1.3% (w/v) agarose gel as described previously (Sambrook et al., 1989). After RNA transfer onto a nylon membrane, the blots were prehybridized, hybridized, and washed according to the method of Moon et al. (1999).

The *OsMADS6*-specific probe (296-bp fragment between nucleotides 748 and 1043) and the *OsMADS14*-specific probe (626-bp fragment between nucleotides 730 and 1355) were labeled by the random priming method (Amersham).

Quantitative Assay of β -Galactosidase Activity

Mid to late exponential-phase yeast cells were collected and resuspended in Z buffer (Miller, 1972). The cells were assayed for β -galactosidase activity as described by Miller (1972) using *O*-nitrophenyl β -D-galactopyranoside as a substrate. The activity unit was calculated using the formula: $1,000 \times A_{420} / (A_{600} \times \text{assay time in minutes} \times \text{assay volume in milliliters})$.

Construction of Phylogenetic Trees

Alignment of conceptual amino acid sequences was made using the Jotun Hein method in the program MegAlign of the DNASTAR (Madison, WI) phylogenetic package. Phylogenetic trees were constructed by comparing 170 amino acid sequences comprising the MADS box, the I region, and the K domain.

RESULTS

Isolation of a Rice cDNA Clone Encoding a MADS Box Protein

A cDNA clone was isolated by screening the Uni-ZAP cDNA library that was prepared from rice floral primordia using the *OsMADS1* cDNA as a probe (Chung et al., 1994). This clone was designated *OsMADS6*. DNA sequence analysis showed that this cDNA clone is 1,043 nucleotides long and encodes a putative protein of 250 amino acid residues (calculated $M_r = 28,400$; accession no. U78782). The MADS box domain of the cDNA clone is located between the 2nd and 57th amino acids of the protein (Fig. 1A). This region is the most conserved region, as observed from other MADS box proteins. The second conserved domain, the K box, is located between the residues 91 and 156.

The *OsMADS6* protein contains two variable regions: the I region between the MADS and K boxes and the C-terminal region downstream of the K box (Purugganan et al., 1995). The C-terminal region of *OsMADS6* has two short motifs, EPTLQIG and AENNFMLGWVL, which are partially conserved in *ZAG3*, *ZAG5*, *AGL2*, *AGL4*, and *AGL9* (Fig. 1A). Based on amino acid sequence similarity of the entire coding region, *OsMADS6* can be grouped into the *AP1/AGL9* family (Purugganan et al., 1995), which includes *AGL2* and *AGL4* of Arabidopsis (Ma et al., 1991), *ZAG3* and *ZAG5*, the maize homolog of *AG* (Mena et al., 1995), floral binding protein 2 (*FBP2*) of petunia (Angenent et al., 1994), *TM5* of tomato (Pnueli et al., 1994), and *OsMADS1*, *OsMADS5*, *OsMADS7*, and *OsMADS8* of rice (Chung et al., 1994; Kang and An, 1997; Kang et al., 1997). Among these genes, *OsMADS6* was the most homologous to *ZAG3* (84%) and *ZAG5* (82%).

Two-Hybrid Screening

We conducted yeast two-hybrid screening to identify proteins that interact with *OsMADS6*. We initially made a fusion between the GAL4 binding domain and the *OsMADS6* protein containing the K domain and the C-terminal region. This fusion molecule (pBD/*OsMADS6*-KC) by itself was able to activate the *HIS3* and β -galactosidase (*LacZ*) selectable reporter genes, which were under the control of the GAL1 and GAL4 upstream activating sequences, respectively. This indicates that the K domain and the C-terminal region carries an activation domain. It was recently observed that other MADS box proteins also carry an activation domain in the C-terminal regions (Moon et al., 1999). Therefore, we made another molecule that was identical to pBD/*OsMADS6*-KC except

A

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MGRGRVELKRIENKINROVTFSKRRNGLLKKAYELSVLQDAEVALIFSSRGKLYEFGSA OsMADS6
*****
*****M*****V
GITKTLERYQHCCYNAQDSNN-ALSETQSWYHENSCLKAKFEALORTORHLLGEDLGPLS ZAG3
*****G*****R*****E*****
*****S*****S*****R*****G*****
**ES*I**NR**CSL**KPEET**CQ*VT**S*Y*S*V*N*****EMG ZAG5
*****
VKELQOLEKOLECALQARQKTKOLMVEQVEELRKRERQJLGEINRQLKHKHLEVEGSTSY AGL6
*****M*****A*-C**
*****T**H**M*****A*-C**
*****A**R**A**TAT*****EM*D**K*****D**K**I**F**T**--HAF
RAMQAS--WAQAVVEN-GAAYVQPPP--HSAAMDSEPTLQIGYP--HQFVPAEAN-T
*TL*H*A-WPAPGSTM*HD*T*HVH*TTAQ*V**C*****PH**I*S*ANN
TTL*H*ACWPAFG*TI*HD**T*QVH**A**V**C*****H**P**V*VNN
KTF*DLWANSA*SV*GDP*-NSEFPVE*SHPNVLDONT**F****FQ-Q*Y*Y*QG*GS-S
IQRSTAPAGAENNEMLGWVL
*p*--*P*G*****
*p*--*A*TC*****
VSK*--NVAG*T**VQ***
    
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B

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MGRGKVLKRIENKINROVTFSKRRSGLLKKANEISVLCDAEVALIFSTKGLYEFYATD OsMADS14
*****N*****H*****V*V*P***** ZAP1
SCMDKILERYERYSYAEKVLISAESDTQGNWCHYEYRKLKAKVETIOKCKOKHLMGEDLESJ
*R*****A*****ESE*****I*****H*****
NLKELQOLEQOLENSLKHRSRKSQJLMEISINELQRKESLQEEKNVLQKELVEKQ---
*p*****DS*****H**A**S**K**R*****A*****A**R*KAVA
---KVQKQVQWDQT---QFQTSSSSSSFMMR---EALP-TTNISNYPAAAGERIEDVAA
SRQQQ*Q*****QTHA*A*****QDQQG**PPH**CFP*LTM*D*G*EL**
G-----QPQHERIGLPPWMLSHING
AAAAQQQPLPGQA**LRIA*****L*A
    
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Figure 1. Deduced amino acid sequence comparisons of MADS box proteins. A, Alignment of the amino acid sequences of OsMADS6, ZAG3, ZAG5, and AGL6. The two conserved motifs of the C region are indicated in bold. B, Alignment of the amino acid sequences of OsMADS14 and ZAP1, an AP1 homolog of maize. The MADS box regions are underlined and the K domains are double underlined. Asterisks indicate identical amino acid residues. Dashes indicate gaps introduced to maximize alignments.

that most of the C-terminal end was deleted, leaving only the 14 amino acid residues of the C region located immediately downstream of the K region. This plasmid, pBD/OsMADS6-KC14, 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 His, demonstrating that the fragment containing the K domain and 14 amino acid

residues of the C region of OsMADS6 does not contain an activator domain.

We therefore proceeded to introduce the cDNA expression library constructed from the mRNA of young rice panicles into the YRG-2 yeast strain containing pBD/OsMADS6-KC14. A total of 1.4×10^6 transformants was screened for their ability to grow on a medium lacking His. This initial screening identified 59 colonies, which were subsequently tested for activation of the *LacZ* gene. These experiments resulted in the identification of 45 colonies that activated both *HIS3* and *LacZ*. Plasmid DNAs were prepared from these colonies and retransferred into the YRG-2 strain to confirm whether the activation is indeed due to the presence of the fusion protein. We observed that 39 plasmids were able to activate the *LacZ* gene only in the presence of pBD/OsMADS6-KC14. Sequence determination of these clones revealed that 38 plasmids contained an ORF that exhibited a significant homology to MADS box proteins (Table I). The remaining plasmid had some homology to MADS genes, but was significantly different from typical plant MADS box genes. This clone was not studied further.

Eleven of the clones encoded for previously identified MADS box proteins; of these, six clones belong to *OsMADS1* (Chung et al., 1994), two to *OsMADS5* (Kang and An, 1997), and three to *OsMADS7* (Kang et al., 1997). The remaining plasmids encode for MADS box proteins not previously reported. Twelve of these were partial clones of an identical MADS box gene, although the 5' ends were different from each other (Fig. 2). This gene was designated *OsMADS14*. Thirteen clones encoded for an identical protein of another MADS box protein. The gene for these clones was designated *OsMADS15*. Both the *OsMADS14* and *OsMADS15* proteins were highly homologous to ZAP1. Among the remaining two clones, one clone, *OsMADS17*, showed a high similarity with ZAG3 and the last clone, *OsMADS18*, was the most homologous to ZAP1.

Isolation of the OsMADS14 cDNA Clone Containing an Entire ORF

All of the twelve cDNA clones of *OsMADS14* selected by the two-hybrid screening were partial, lacking the 5' region that encodes for the N-terminal end of the protein (Fig. 2). The 5' region was isolated by PCR using a vector primer and the cDNA specific primer. A cDNA clone of 1,355 bp, containing the entire ORF, was generated by connecting the 5' region to the cDNA clone obtained from the two-

Table I. The MADS proteins isolated by yeast two-hybrid screening using OsMADS6 as bait

Gene Name	No. of Isolated Clone(s)	Homologous Gene	Reference
<i>OsMADS1</i>	6	<i>AGL2</i>	Chung et al. (1994)
<i>OsMADS5</i>	2	<i>AGL2</i>	Kang and An (1997)
<i>OsMADS7</i>	3	<i>AGL2</i>	Kang et al. (1997)
<i>OsMADS14</i>	12	<i>ZAP1</i>	This study
<i>OsMADS15</i>	13	<i>ZAP1</i>	This study
<i>OsMADS17</i>	1	<i>ZAG3</i>	This study
<i>OsMADS18</i>	1	<i>ZAP1</i>	This study

CT CGA TGG ATC CAT CCA TGG ATC GAT GGG TCC CGC CCA CCG CGC GCG ACG CAT TCC GCC 59
 GCC GTC TGG CCG TGT CCA CGT GAT GGG CGC GGC TAG GGG ATA GGC GGA TAG CCA GCA 119
 GCC ACC ACC ACC AGT AGT TGC CGT GTG GGG ATA GGT GTG GCT ATA GGG CTA GTG GTC GTC 179
 GCT GAT AGC GAG GTG GGT AGG GTT AAT TTT GGT TGG AGG TAG AGA GAG AGA GAG AGG GAG 239
 GGA GGG AGG AGG AGG AGG AGG AGG AGG AGG AGG AGG AAC AGG AGG AAG ATG GGG CGG GGC 299
 M G R G 4

AAG GTG CAG CTG AAG CCG ATC GAG AAC AAG ATC AAC CCG CAG GTG ACC TTC TCC AAG CGC 359
 K V Q L K R I E N K I N R Q V T F S K R 24

AGG TGG GGG CTG CTC AAG AAG GCG AAT GAG ATC TCC GTG CTC TGC GAC GCC GAG GTC GCG 419
 R S G L L K K A N E I S V L C D A E V A 44

CTC ATC ATC TTC TCC ACC AAG GGC AAG CTC TAC GAG TAC GCC ACC GAC TCA TGT ATG GAC 479
 L I I F S T K G K L Y E Y A T D S C M D 64

AAA ATC CTT GAA CGT TAT GAG CGC TAC TCC TAT GCA GAA AAG GTC CTT ATT TCA GCT GAA 539
 K I L E R Y E R Y S Y A E K V L I S A E 84

TCT GAC ACT CAG GGC AAC TGG TGC CAC GAA TAT AGG AAA CTG AAG GCT AAG GTT GAG ACA 599
 S D T Q G N W C H E Y R K L K A K V E T 104

ATA CAG AAA TGT CAA AAG CAC CTC ATG GGA GAG GAT CTT GAA TCT TTG AAT CTC AAA GAG 659
 I Q K C Q K H L M G E D L E S L N L K E 124

CTG CAG CAG CTG GAG CAG CAG CTG GAA AAT TCG TTG AAA CAT ATC AGA TTC AGA AAG AGC 719
 L Q Q L E Q Q L E N S L K H I R F R K S 144

CAA CTA ATG CTC GAG TCC ATT AAC GAG CTT CAA CGG AAG GAA AAG TCA CTG CAG GAG GAG 779
 Q L M L E S I N E L Q R K E K S L Q E E 164

AAT AAG GTC CTA GAG AAA GAA CTG GTG GAG AAG CAG AAA GTC CAG AAG CAA CAA GTG CAA 839
 N K V L Q K E E L V E K Q K V Q K Q Q Y Q 184

TGG GAC CAG ACA CAA CCT CAA ACA AGT TCC TCA TCA TCC TCC TTC ATG ATG AGG GAA GCC 899
 W D Q T Q P Q T S S S S S S F M M R E A 204

CTT CCA ACA ACT AAT ATC AGT AAC TAC CCT CCA GCA GCT GGC GAA AGG ATA GAG GAT GTA 959
 L P T T N I S N Y P A A A G E R I E D V 224

GCA GCA GGG CAG CCA CAG CAT GAA CGC ATT GGG CTG CCA CCA TGG ATG CTG AGC CAC ATC 1019
 A A G Q P Q H E R I G L P P W M L S H I 244

AAC GGC TAA GGA GGC TTC GAG TCC ATA CCA GTA ATC ACA AGT TGC AAC CTG ACC CGG TCC 1079
 N G * 246

GGT CGC CTG CTG CTC TGG TTT ACT ACT AGT ACT ATT GTC ATC TTG CGG TTG CGA GAC GAG 1139
 GAA AGC ATT TTA GCC CTA AAT TCA GCA TTA GTA GCA AGC TGC AAT GTG TAT ATT TTG GCT 1199
 TCG TCC ACC ACC GTC TTC CTC CCA CCA GTA ATT TAC CCA TGT AAT ATA TGC GAG CAG CAT 1259
 GAA CAA ATT TTC CGT TTT CCA ACC ATC TCC ATT GGT GTC ATG TGT GAC TTA AAT AGC GAA 1319
 ATT TCA CCA TTG TGC ATA GTG TGA TTA CTG TAA GAT 1355

Figure 2. The nucleotide sequence and the deduced amino acid sequence of *OsMADS14*. The MADS box region is underlined, and the K domain is double underlined. The 10 repeats of the GGA sequence in the 5'-UTR are indicated in bold type. The primer sequence used in isolation of the 5' region of the gene is underlined. Arrowheads and numbers below the amino acid sequence indicate positions of the first amino acid of the fusion proteins selected by the yeast two-hybrid screening and the number of selected clones with the same first amino acid, respectively. The *XhoI* site used for generation of the gene-specific probe (accession no. AF058697) is indicated in bold type.

hybrid screening. It contains a 287-bp 5'-UTR and an ORF of 246 amino acid residues (calculated $M_r = 28,500$; accession no. AF058697).

The 5' UTR of *OsMADS14* cDNA contains 10 repeats of the GGA sequence (Fig. 2), and such repeat sequences were previously observed from other rice MADS-box genes (Chung et al., 1994; Kang and An, 1997; Kang et al., 1997). The *OsMADS14* protein contains a MADS box domain which consists of 56 conserved amino acids present in the N-terminal region of all of the MADS transcription factors (Figs. 1B and 2). The K box domain, a region considered to participate in the protein-to-protein interaction, is also present between amino acid residues 91 and 158. Amino

acid sequence comparison revealed that *OsMADS14* was 72.4% homologous to ZAP1, an AP1 homolog of maize.

Expression Patterns of *OsMADS6* and *OsMADS14*

It has been well established that there are a large number of MADS box genes in the rice genome (Chung et al., 1994). Therefore, it was necessary to identify the region that does not cross hybridize with other MADS box genes by genomic DNA blot analyses. It was observed that the 300-bp *PstI-EcoRI* fragment located at the C-terminal region of *OsMADS6* hybridized to a single DNA fragment (Fig. 3A). Likewise, the 630-bp *XhoI* fragment of *OsMADS14* was shown to be a gene-specific region. In genomic DNA analysis of *OsMADS14*, three *PstI* fragments were hybridized with the probe (Fig. 3A), due to the presence of two *PstI* sites in the region used for the probe.

RNA blot analyses were conducted using the gene-specific probes. The results showed that the *OsMADS6* transcript was detectable primarily in lodicules and also weakly in sterile lemmas and carpels of flowers at the late vacuolated pollen stage (Fig. 3B). However, the transcript was not detectable in stamens, paleas/lemmas, and vegetative organs. The spatial expression pattern of *OsMADS14* was different from that of *OsMADS6*. Transcripts of this

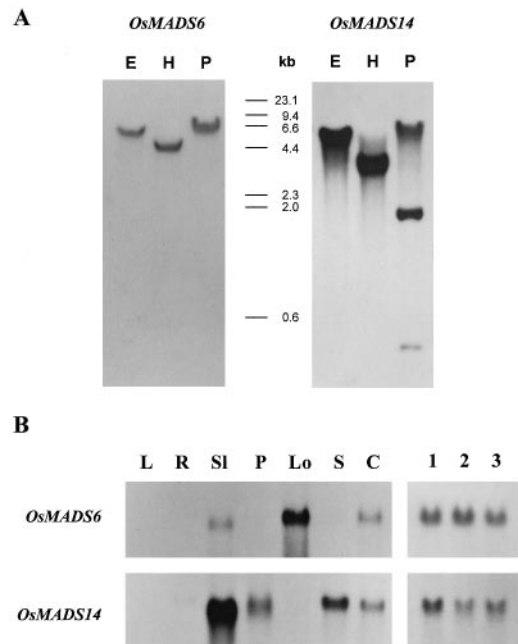


Figure 3. Genomic DNA blot analysis and RNA blot analysis of *OsMADS6* and *OsMAD14*. A, Southern blot analysis of *OsMADS6* and *OsMAD14*. The rice genomic DNA was digested with *EcoRI* (E), *HindIII* (H), or *PstI* (P). The numbers indicate the size (in kb) of the DNA markers. B, RNA blot analysis of *OsMADS6* and *OsMAD14*. Ethidium bromide staining of 25S and 17S rRNAs demonstrated equal amounts of RNA loading (data not shown). Lane L, Leaves; lane R, roots; lane Sl, sterile lemmas; lane P, paleas/lemmas; lane Lo, lodicules; lane S, stamens; lane C, carpels; lane 1, young flowers with a panicle size of 1 to 5 cm; lane 2, flowers at the early vacuolated pollen stage; lane 3, flowers at the late vacuolated pollen stage.

clone were detectable primarily in sterile lemmas and also weakly in paleas/lemmas, stamens, and carpels (Fig. 3B). However, the *OsMADS14* transcripts were not detected in lodicules and vegetative organs. During flower development the *OsMADS6* and *OsMADS14* genes were expressed at an early stage, and their expression was extended into later stages of flower development (Fig. 3B).

Interaction between OsMADS6 and Other MADS Box Proteins

The yeast two-hybrid screening resulted in identification of seven types of MADS box proteins that interact with OsMADS6. To confirm these results, we investigated the protein-to-protein interaction between OsMADS6 and other rice MADS box proteins. The C-terminal half containing the K domain and C region of *OsMADS1*, *OsMADS3*, *OsMADS4*, *OsMADS5*, *OsMADS6*, *OsMADS7*, *OsMADS8*, *OsMADS14*, *OsMADS15*, *OsMADS17*, and *OsMADS18* was fused to the activation domain of *GAL4* using the pAD-GAL4 vector. These plasmids were introduced into the yeast strain YRG-2 containing the binding domain plasmid pBD/OsMADS6-KC14. The colonies that grew on a medium lacking Leu and Trp were examined for β -galactosidase activity. The results in Table II show that the KC regions of OsMADS1, OsMADS5, OsMADS7, OsMADS8, OsMADS14, OsMADS15, OsMADS17, and OsMADS18 were able to activate the *LacZ* gene. However, the KC regions of OsMADS3, OsMADS4, and OsMADS6 did not activate the reporter gene. Western blot analyses showed that the β -galactosidase protein level was proportional to the enzyme activity (data not shown).

To confirm the lack of interaction, the region containing the K domain and 14 amino acids of the C-terminal region of the OsMADS3 and OsMADS4 proteins were fused to the binding domain vector pBDGAL4. Most of the C-terminal regions of OsMADS3 and OsMADS4 were not included in the construction to avoid a potential activator domain. Introduction of these plasmids into the YRG-2 strain con-

taining pAD/OsMADS6-KC did not activate the *LacZ* gene (data not shown). These results showed that OsMADS6 interacts with OsMADS1, 5, 7, 8, 14, 15, 17, and 18, members of the AP1/AGL9 family, but not to the B and C class of MADS box proteins, OsMADS4 and OsMADS3; it also failed to interact with OsMADS6 itself.

Identification of the Protein-to-Protein Interaction Motif

The motif responsible for the protein-to-protein interaction between OsMADS6 and OsMADS14 was investigated using the yeast two-hybrid system. The MADS box domain and the I region (MI), the K domain and 14 amino acid residues of the C-region (KC14), the MI and KC14 region (MIKC14), and the K domain (K) of OsMADS6 were connected to the activation domain and the binding domain of GAL4. The K domain and C-terminal region (KC) of OsMADS6 was fused to the activation domain of GAL4. Similarly, the MI region, the MIKC14 region, and the KC14 region of OsMADS14 were connected to the activation domain and the binding domain of GAL4, and the KC region of OsMADS14 to the activation domain. The transformants that grew on a medium lacking Leu and Trp were examined for activation of the *LacZ* gene by a β -galactosidase activity analysis (Table III). When pAD/OsMADS6-K and pBD/OsMADS14-KC14 were introduced into YRG-2, the *LacZ* gene was activated. Similarly, pBD/OsMADS6-K and pAD/OsMADS14-KC activated the reporter gene expression. Moreover, OsMADS6-MIKC14 activated the *LacZ* gene in the presence of OsMADS14-MIKC14. However, the C region by itself containing amino acids 171 to 250 of OsMADS6 did not activate the *LacZ* gene in the presence of OsMADS14-KC (data not shown). Furthermore, OsMADS6-MI and OsMADS14-MI did not activate the *LacZ* gene (Table III). These results suggest that the K box is primarily responsible for heterodimerization between OsMADS6 and OsMADS14. Interestingly, including the 14 amino acid residues immediately downstream of the K box enhanced the enzyme activity by 5- or 20-fold (Table III), suggesting that the 14 residues stabilized or enhanced the interaction in the yeast two-hybrid system.

To narrow down the motif responsible for the protein-to-protein interaction, the KC14 region of OsMADS6 was divided into three regions; KI (86th to 110th amino acid), KII (109th to 137th amino acid), and KIII (138th to 170th amino acid) (Fig. 4). The three regions were connected into the binding domain of GAL4 and introduced into the yeast strain YRG-2 containing pAD/OsMADS14-KC. The results in Table III show that only the KII region activated the *LacZ* gene, suggesting that this region plays an important role in the protein-to-protein interaction. The experiment also indicated that the KIII region alone did not bind to OsMADS14, but enhanced the interaction between these proteins. When pAD/OsMADS14-KC14 was used as the activation domain plasmid instead of pAD/OsMADS14-KC, similar results were observed, although the β -galactosidase activities generally decreased (data not shown). Also, when those regions of OsMADS6 were fused into the activation domain and OsMADS14 was connected to the binding domain, similar results were obtained, except that pAD/OsMADS6-KII did

Table II. Quantitative assay of protein-to-protein interaction between *OsMADS6* and rice MADS proteins

Activation Domain Plasmid	β -Galactosidase Activity ^a	
	pBD/OsMADS6-KC14 ^b	pBDGAL4 ^c
pAD/OsMADS1-KC	88.16 ± 1.357	0.04 ± 0.011
pAD/OsMADS3-KC	0.10 ± 0.014	0.04 ± 0.013
pAD/OsMADS4-KC	0.06 ± 0.012	0.04 ± 0.013
pAD/OsMADS5-KC	67.99 ± 2.096	0.05 ± 0.014
pAD/OsMADS6-KC	0.29 ± 0.023	0.03 ± 0.012
pAD/OsMADS7-KC	39.04 ± 3.417	0.04 ± 0.013
pAD/OsMADS8-KC	17.91 ± 0.631	0.04 ± 0.011
pAD/OsMADS14-KC	84.66 ± 4.389	0.02 ± 0.006
pAD/OsMADS15-KC	44.83 ± 1.937	0.02 ± 0.006
pAD/OsMADS17-KC	39.58 ± 3.619	0.04 ± 0.011
pAD/OsMADS18-KC	52.32 ± 2.175	0.04 ± 0.013
None	0.09 ± 0.032	0.04 ± 0.013

^a β -Galactosidase activity unit = $1,000 \times A_{420}/[A_{600} \times \text{reaction time (min)} \times \text{volume of culture (mL)}]$. ^b GAL4 DNA binding domain fusion protein. ^c pBDGAL4 is used as a negative control.

Table III. Investigation of the motif responsible for protein-to-protein interaction between *OsMADS6* and *OsMADS14*

Activation Domain Plasmid	Binding Domain Plasmid	β -Galactosidase Activity ^a
pAD/OsMADS14-MIKC14	pBD/OsMADS6-MIKC14	10.21 ± 1.007
pAD/OsMADS14-MIKC14	pBD/OsMADS6-MI	0.09 ± 0.011
pAD/OsMADS14-MI	pBD/OsMADS6-MIKC14	0.06 ± 0.007
pAD/OsMADS14-MI	pBD/OsMADS6-MI	0.07 ± 0.032
pAD/OsMADS14-KC	pBD/OsMADS6-K	13.22 ± 1.310
pAD/OsMADS14-KC	pBD/OsMADS6-KC14	62.12 ± 7.583
pAD/OsMADS14-KC	pBD/OsMADS6-KI	0.14 ± 0.015
pAD/OsMADS14-KC	pBD/OsMADS6-KII	18.21 ± 3.224
pAD/OsMADS14-KC	pBD/OsMADS6-KIII	0.16 ± 0.023
pAD/OsMADS14-KC	pBD/OsMADS6-KII+KIII	53.40 ± 2.824
pAD/OsMADS6-MIKC14	pBD/OsMADS14-MIKC14	8.14 ± 0.075
pAD/OsMADS6-MI	pBD/OsMADS14-MIKC14	0.08 ± 0.010
pAD/OsMADS6-MIKC14	pBD/OsMADS14-MI	0.06 ± 0.011
pAD/OsMADS6-MI	pBD/OsMADS14-MI	0.05 ± 0.008
pAD/OsMADS6-K	pBD/OsMADS14-KC14	3.21 ± 1.024
pAD/OsMADS6-KC14	pBD/OsMADS14-KC14	61.23 ± 7.972
pAD/OsMADS6-KC	pBD/OsMADS14-KC14	59.14 ± 4.927
pAD/OsMADS6-KI	pBD/OsMADS14-KC14	0.04 ± 0.007
pAD/OsMADS6-KII	pBD/OsMADS14-KC14	0.06 ± 0.032
pAD/OsMADS6-KIII	pBD/OsMADS14-KC14	0.06 ± 0.016
pAD/OsMADS6-KII+KIII	pBD/OsMADS14-KC14	17.43 ± 2.972

^a β -Galactosidase activity unit = $1,000 \times A_{420}/[A_{600} \times \text{reaction time (min)} \times \text{volume of culture (mL)}]$.

not activate the *LacZ* gene in the presence of pBD/OsMADS14-KC14 (Table III). None of the activation domains or binding domain plasmids used in these experiments activated the *LacZ* gene by itself (data not shown).

Identification of the Amino Acid Residues Responsible for the Protein-to-Protein Interaction

Because it was determined that the KII and KIII regions containing amino acids 109 to 170 of OsMADS6 play an important role in the protein-to-protein interaction between OsMADS6 and OsMADS14, we investigated the amino acid residues responsible for the interaction. The KII and KIII regions of OsMADS6 have periodical and conserved Leu residues (Fig. 4). It has been previously suggested that such hydrophobic repeats may be involved in protein-to-protein interactions (Ma et al., 1991; Pnueli et al.,

1991; Theissen et al., 1995). Therefore, we made the following five mutant fragments: OsMADS6-KIIS₁₁₀R₁₁₈: replacement of Leu-110 residue with Ser and Leu-118 with Arg in the KII fragment; OsMADS6-KIIR₁₂₆R₁₃₄: replacement of Leu-126 and Leu-134 Leus with Arg residues in the KII fragment; OsMADS6-KIIS₁₁₀R₁₁₈+KIII: replacement of Leu-110 with Ser and Leu-118 with Arg in the KII+KIII fragment; OsMADS6-KII+KIIR₁₅₉R₁₆₆: replacement of Leu-159 and Leu-166 with Arg residues in the KII+KIII fragment; and OsMADS6-KIIS₁₁₀R₁₁₈+KIIR₁₅₉R₁₆₆: replacement of Leu-110 with Ser and Leu-118, Leu-159, and Leu-166 Leus with Arg residues in the KII+KIII fragment.

These mutant fragments were connected to the binding domain of GAL4 and transferred into the YRG-2 strain containing the activation domain plasmid pAD/OsMADS14-KC. The results in Table IV show that mutations in Leu-110 and Leu-118 diminished the interacting

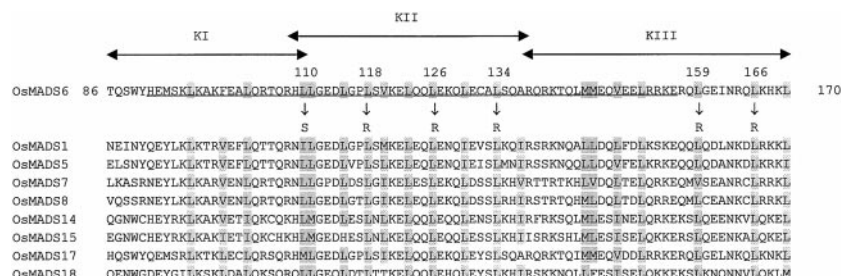


Figure 4. Amino acid sequence alignment of the region containing the K domain of OsMADS6 with those of OsMADS1, OsMADS5, OsMADS7, OsMADS8, OsMADS14, OsMADS15, OsMADS17, and OsMADS18. The region of OsMADS6 was divided into three regions: the KI region (amino acids 86–110), the KII region (amino acids 109–137), and the KIII region (amino acids 138–170). The entire K domain of OsMADS6 is underlined. The replaced amino acids are indicated below each Leu with arrows. The conserved hydrophobic residues, such as Leu, Ile, Val, and Met, are shaded. The numbers indicate the positions of mutagenized Leu residues and the first and last amino acids of the K region elucidated in this study.

Table IV. Investigation of the amino acid residues responsible for the interaction between *OsMADS6* and *OsMADS14* by site-directed mutagenesis of amino acids in the K domain and 14 amino acids of the C region of *OsMADS6*

Activation Domain Plasmid	Binding Domain Plasmid	β -Galactosidase Activity ^a
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KII	10.52 \pm 2.460
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈	0.17 \pm 0.016
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KIIR ₁₂₆ R ₁₃₄	0.05 \pm 0.015
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KII+KIII	50.63 \pm 1.983
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈ +KIII	29.33 \pm 2.660
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈ +KIIR ₁₅₉ R ₁₆₆	0.07 \pm 0.012
pAD/ <i>OsMADS14</i> -KC	pBD/ <i>OsMADS6</i> -KII+KIIR ₁₅₉ R ₁₆₆	0.07 \pm 0.016
pAD/ <i>OsMADS6</i> -KII	pBD/ <i>OsMADS14</i> -KC14	0.09 \pm 0.027
pAD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈	pBD/ <i>OsMADS14</i> -KC14	0.08 \pm 0.032
pAD/ <i>OsMADS6</i> -KIIR ₁₂₆ R ₁₃₄	pBD/ <i>OsMADS14</i> -KC14	0.08 \pm 0.016
pAD/ <i>OsMADS6</i> -KII+KIII	pBD/ <i>OsMADS14</i> -KC14	13.91 \pm 0.905
pAD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈ +KIII	pBD/ <i>OsMADS14</i> -KC14	3.11 \pm 0.247
pAD/ <i>OsMADS6</i> -KIIS ₁₁₀ R ₁₁₈ +KIIR ₁₅₉ R ₁₆₆	pBD/ <i>OsMADS14</i> -KC14	0.09 \pm 0.007
pAD/ <i>OsMADS6</i> -KII+KIIR ₁₅₉ R ₁₆₆	pBD/ <i>OsMADS14</i> -KC14	0.09 \pm 0.013

^a β -Galactosidase activity unit = 1,000 \times $A_{420}/[A_{600} \times$ reaction time (min) \times volume of culture (mL)].

ability of the KII fragment. Similarly, mutations of Leu-126 and Leu-134 also significantly affected the activity. These results suggest that the four periodical Leus in the KII region are necessary for interaction between the K box regions. When the mutations at Leu-110 and Leu-118 were introduced into the KII+KIII fragment, the enzyme activity was reduced, but still retained a significant level of activity. However, when mutations were introduced into both KII and KIII regions by replacing Leu-110, Leu-118, Leu-159, and Leu-166, the activity was almost completely diminished. These results, together with the results shown in Table III, indicate that the KIII region alone is not sufficient for protein interaction, but is able to enhance the interaction between MADS box proteins.

When the mutant fragments were used in the construction of activation domain plasmids and their interaction ability in the yeast strain carrying the binding domain plasmid pBD/*OsMADS14*-KC14 was tested, similar results were obtained except that pAD/*OsMADS6*-KII and pBD/*OsMADS14*-KC14 did not activate the *LacZ* gene (Table IV). Taken together, these results suggest that the Leu residues in the KII region are important for the protein interaction

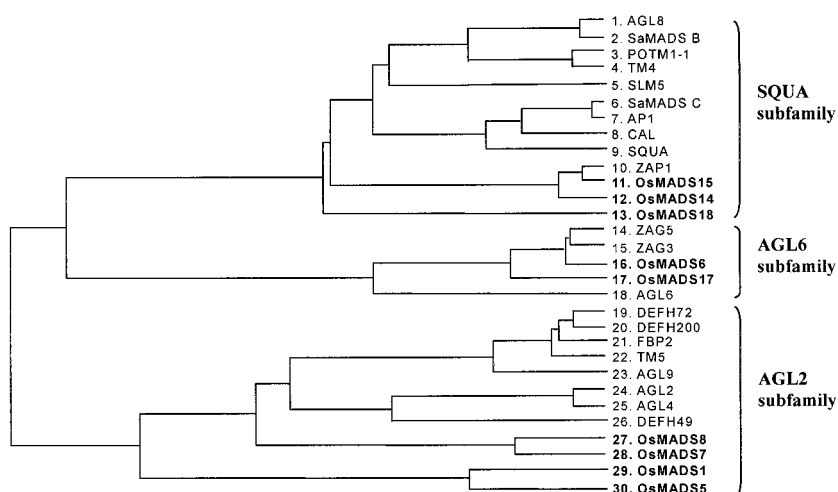
and the Leu residues in the KIII region are involved in enhancing the interaction.

DISCUSSION

We have isolated the *OsMADS6* gene by screening a young rice flower library using *OsMADS1* as a probe. On the basis of deduced amino acid sequences, the *OsMADS6* gene can be classified into the *AGL6* subfamily (Theissen et al., 1996), which belongs to the *AP1/AGL9* family (Purugganan et al., 1995) (Fig. 5). In addition, we have isolated various MADS box genes that encode for protein-to-protein interaction partners of *OsMADS6* by the yeast two-hybrid screening method.

It was observed that the region containing the K domain and the C-terminal region of *OsMADS6* had transcription activation ability in yeast. The region responsible for the activation appears to be located in the C-terminal region, since deletion of the region eliminated the activation ability. Recently, we reported that *OsMADS16*, a rice AP3 homolog, contains a motif responsible for the transcription activation ability in the C-terminal region (Moon et al.,

Figure 5. Phylogenetic tree showing the relationship among AP1/AGL9 family proteins. Rice MADS box proteins are indicated in bold type. The horizontal branches are proportional to the number of base substitutions. 1, 7, 8, 18, 23, 24, and 25, *Arabidopsis*; 9, 19, 20, and 26, snapdragon; 10, 14, and 15, maize; 11, 12, 13, 16, 17, 27, 28, 29, and 30, rice; 21, petunia; 3, potato; 4 and 22, tomato; 5, *Silene latifolia*; 2 and 6, *Sinapis alba*.



1999). We also observed the transcription activation ability of other rice MADS box proteins such as *OsMADS1*, *OsMADS3*, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *OsMADS14* (unpublished data), and found that one of the roles of the C-terminal region of MADS box proteins is transcription activation.

Almost all of the genes isolated by two-hybrid screening using *OsMADS6* corresponded to MADS box proteins. This suggests that MADS box proteins efficiently interact with each other in yeast. The isolated clones belonged to seven MADS genes that can be classified into three groups in the *AP1/AGL9* family (Table I; Fig. 5). The *OsMADS1*, *OsMADS5*, and *OsMADS7* genes belong to the *AGL2* subfamily, the *OsMADS17* gene belongs to the *AGL6* subfamily, and the *OsMADS14*, *OsMADS15*, and *OsMADS18* genes belong to the *SQUA* subfamily (Theissen et al., 1996). In addition to these seven MADS box proteins, *OsMADS6* also interacted with *OsMADS8*, which is also a member of the *AGL2* subfamily (Kang et al., 1997). However, *OsMADS6* did not interact with *OsMADS3* and *OsMADS4*, rice homologs of *AG* and *PI*, respectively (Table II). In addition, *OsMADS6* failed to interact with itself.

In studies using the yeast two-hybrid system, it was previously reported that *AG*, a C-class protein in Arabidopsis, interacted with *AP1/AGL9* family proteins such as *AGL2*, *AGL4*, *AGL6*, and *AGL9*, most of which are expressed at the early flowering stage (Fan et al., 1997). Moreover, MADS box proteins expressed at the early (*SQUA*), intermediate (*DEFH200* and *DEFH72*), and late (*DEFH49*) stages of flower development were also identified as interaction partners of *PLE*, a C-class MADS box protein in snapdragon, by the two-hybrid screening method (Davies et al., 1996). Sequence similarity and expression patterns showed that *DEFH200* and *DEFH72* were very similar to *FBP2* and *TM5*, and *DEFH49* was very similar to *AGL2*. These results indicate that C-class proteins and *AP1/AGL9* family proteins interact with each other in Arabidopsis and snapdragon. However, we demonstrated that in rice the *AP1/AGL9* family proteins interact with each other within the family, but do not interact with *OsMADS3*, a C-class protein.

The temporal expression patterns of the rice *AP1/AGL9* family genes, such as *OsMADS1*, *OsMADS5*, *OsMADS6*, *OsMADS7*, *OsMADS8*, and *OsMADS14*, were similar (Chung et al., 1994; Kang and An, 1997; Kang et al., 1997). These six MADS box genes were expressed at the early stage of the flower development and their expressions were extended into later stages of flower development. However, the spatial expression patterns of these MADS box genes were different. In mature flowers the *OsMADS1* transcript was present in paleas/lemmas and carpels (Chung et al., 1994), *OsMADS5* in anthers and weakly in carpels (Kang and An, 1997), *OsMADS7* and *OsMADS8* in carpels and weakly in anthers (Kang et al., 1997), *OsMADS14* in sterile lemmas, paleas/lemmas, stamens, and carpels, and *OsMADS6* in lodicules, sterile lemmas, and carpels (Fig. 3B). It is likely that an *AP1/AGL9* protein cooperates with another *AP1/AGL9* protein coexisting in specific cell types at a particular developmental stage.

Ectopic expression of *OsMADS6* and *OsMADS14* in rice exhibited extreme early flowering and dwarfism, which were also observed in rice plants ectopically expressing *OsMADS1*, *OsMADS5*, *OsMADS7*, and *OsMADS8* (unpublished results). However, the early flowering and dwarfism in transgenic plants of those four MADS genes was not as extreme as in those of *OsMADS6* and *OsMADS14*, indicating that *OsMADS6* and *OsMADS14* might regulate a very early stage of flower development. Further *in vivo* studies are now being undertaken to identify whether a heterodimer between *OsMADS6* and its interaction partners participates in these developmental processes.

It is known that MADS box proteins interact with each other to form dimers (Davies et al., 1996; Fan et al., 1997). In the present study, motifs responsible for the interaction between *OsMADS14* and *OsMADS6* proteins were investigated using the yeast two-hybrid screening method. Our results showed that the K domain plays a very important role in the protein-to-protein interaction. In Arabidopsis, it was previously reported that *AG* forms a dimer with various *AGL* proteins via K-domain-mediated interaction (Fan et al., 1997). It was also reported that the K domain was essential for protein-to-protein interaction between *GLO* and *DEF* (Davies et al., 1996).

It has also been suggested that the *AG* and *AGL* proteins contain two helices in the K domain, which may participate in the protein-to-protein interaction (Ma et al., 1991). The KI and KII regions of *OsMADS6* contain one helix each. In the present study, we demonstrated that the KII region carrying the second helix plays a more important role in the interaction. The site-directed mutation analysis revealed that Leu-126 and Leu-134 of the KII region play an important role in the interaction. These amino acids are the hydrophobic residues suggested to cluster on one face of the putative helices (Theissen et al., 1995). Therefore, it can be proposed that the putative helix structure plays an important role in the K-domain-mediated interaction between *OsMADS14* and *OsMADS6*.

In Arabidopsis, it was reported that the C region of the *AG* and *AGL* proteins enhanced the K-domain-mediated interaction between these proteins (Fan et al., 1997). In the present study, we showed that the 14 amino acid residues located immediately downstream of the K domain enhanced the interaction between *OsMADS6* and *OsMADS14*. In Arabidopsis, *ag-4* mutants that result from deletions of 12 or 14 amino acids downstream of the K domain retain a partial *AG* activity (Sieburth et al., 1995). Therefore, it appears that the region is not essential for function, but is necessary for full activity, probably by enhancing the interaction between *AG* and its partner. The deleted amino acids correspond to the residues between amino acids 142 and 153 or 142 and 155 of *OsMADS6*, which are located in the KIII region.

We demonstrated by site-directed mutagenesis experiments that Leu-110, Leu-118, Leu-126, and Leu-134 in the KII region and at Leu-159 and Leu-166 residues in the KIII region of *OsMADS6* play a very important role in the protein interaction. These periodical hydrophobic residues are conserved in *OsMADS6* and its interaction partners such as *OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8*,

OsMADS14, OsMADS15, OsMADS17, and OsMADS18 (Fig. 4), supporting the idea that these amino acids are important for the function of MADS box proteins.

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