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# Class A and class B MADS box genes for rice flower development

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**Abstract**

We have previously isolated *OsMADS4* gene that is a member of the class B MADS box genes from rice. In this study, another member of the class B MADS box genes was isolated from rice flower by the yeast two-hybrid screening method using OsMADS4 as bait. RNA blot analyses revealed that the clone, *OsMADS16*, 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 with those of *AP3* and *PI*, the class B genes of Arabidopsis, 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. We have also isolated *OsMADS6* gene using *OsMADS1* as a probe. Both are members of the AGL2 MADS family. Various MADS genes that encode for protein-protein interaction partners of the OsMADS6 protein were isolated by the yeast two-hybrid screening method. A majority of these genes belong to the AGL2 family. Sequence homology, expression pattern, and ectopic expression phenotypes indicated that one of the interaction partners, *OsMADS14*, appears to be homologous to AP1, the class A MADS gene of Arabidopsis.

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 [35]. It has been established that three classes of homeotic genes, A, B, and C, which encode proteins containing a highly conserved 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* (*AP1*) in *Arabidopsis* [8, 21] and *SQUAMOSA* (*SQUA*) in *Antirrhinum* [10], the class B genes include *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* [7, 12] and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum* [34], and the class C genes include *AGAMOUS* (*AG*) in *Arabidopsis* [36] and *PLENA* (*PLE*) in *Antirrhinum* [2]. In addition to specification of stamen and carpel identity, the class C genes inhibit the indeterminate growth of the floral meristem [24].

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 [19]. Also, simultaneous expression of both *DEF* and *GLO* in tobacco plants caused extreme alterations of the floral organs [6]. 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 [9, 28, 33]. It was reported that heterodimerization between AP3 and PI was necessary for nuclear localization of these proteins [22]. *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 [28]. In addition, it was demonstrated that

**A**

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  1    MGRGKIEIKRIKNATNRQVTYSKRRTGIMKKARELTVLCDAQVAIIMFSS
 51    TGKYHEFCSPSTDIKGIFDRYQQAIGTSLWIEQYENMQRTLSHLKDINRN
101    LRTEIRQRMGEDLDGLEFDELRGLEQNVDAALKEVRHRKYHVISTQTETY
151    KKKVKHSYEAYKTLQQELGLCEEPAWFVDNTGGGWDGGAGAGAAADMFAF
201    RVVPSQPNLHGMAYGGNHDLRLA*
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**B**

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AADMFAFRVVPS----QPN--LHGM-A--YGGNHDLRLA        223   OsMADS16
GPNIFAFRLQPS----QPN--LHN--GGG-YNCHDLRLA        228   PnAP3-2
VHNLYAFRLQPLH----PN--LQNE--GG-FGSRDLRLS        222   TM6
                              YGxHDLRLA              PaleoAP3 Motif

GSRAYALRFHQNHHHYYPNHGLHAPSAS--DIITFHLLE        232   AP3
------LRFHQNHHHHYPNHALHEASAS--DIITFHLLE        224   BobAP3
GPRIIALRLPTNHH---PT--LH--SGGGSDLTTFALLE        227   DEF
                              D(L/I)ITTFALLE         EuAP3 Motif
     FxFRLQPSQPNLH                                   PI Motif-derived

S-MPFTFRVQPSHPNLQQEK---                        210   OsMADS4
-QMPFGFQVPPMQPNLTTVTTTTTTNNK                   231   pnPI-1
-Q—FGYRVQPIQPNLQEKIMSLVID                      208   PI
-QMPFAFRVQPMQPNLQERF---                        215   GLO
  MPFxFRVQPxQPNLQE                                   PI-Motif
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*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 a member of the *AP3* family genes of *Papaver nudicaule*, tomato, *Arabidopsis thaliana*, *Brassica oleraceae*, 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 grey-shaded background denotes amino acid sequences of paleoAP3 motif, euAP3 motif, PI motif-derived motif, or PI motif.

the MADS box and I-region are involved in nuclear localization [22]. 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 [5]. 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 [11], 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 [7, 12, 13, 29, 30]. 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 [18]. 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* [4]. 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 [16]. 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.

### *Isolation of OsMADS16*

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

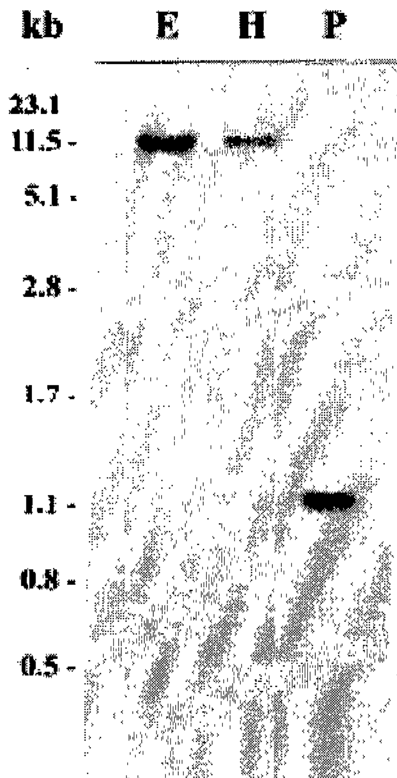


*Figure 2.* Southern blot analysis of *OsMADS16*. The rice genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P), respectively. The numbers indicate the size, in kb, of DNA markers.

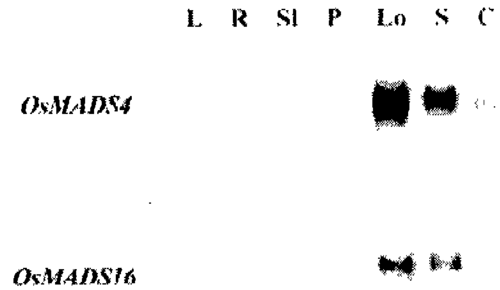


*Figure 3.* RNA blot analysis of *OsMADS4* and *OsMADS16*. Ethidium bromide staining of 25S and 17S rRNAs demonstrated equal amounts of RNA loading (data not shown). L; leaves, R; roots, Sl; sterile lemma, P; palea/lemma, Lo; lodicules, S; stamens, C; carpels.

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 x $10^6$ transformants was 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 OsMADS16 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 for 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 1,059 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 weight = 25.4 kD) (Genbank access No. AF077760). The gene corresponding to this clone was designated *OsMADS16*. The OsMADS16 protein shows 56.6% identity with PnAP3-2, an *AP3* ortholog of *Papaver nudicaule* [18], 49.8% with DEF [31], and 49.3% with AP3 [12]. 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). K box domain, a region considered to participate in protein-protein interaction, is also present in between amino acid residues 89-154. In the C-terminal region, two consensus amino acid sequences are located (Figure 1). One is the PI motif, FxFRLQPSQPNLH, 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 [18].

*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 x $OD_{420}$/[$OD_{600}$ x reaction time (min) x volume of culture (mL)].
[b]GAL4 DNA binding domain fusion protein.
[c]pBDGAL4 is used as a negative control.

*Expression patterns of OsMADS4 and OsMADS16*

It was well established that there are a large number of MADS genes in the rice genome [3, 4, 14, 15, 17]. 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 to 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 *OsMADS16* transcript was present in lodicules and stamens, but not detectable in other organs (Figure 3). Lodicules are small, oval, and fleshy organs located immediately outside of the stamens and are considered to be petal [37]. We have previously shown that the *OsMADS4* gene is expressed specifically in stamens and carpels [16]. 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 [7, 12].

*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. 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

*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 ± 1.957 |
| PI-KC | AP3-KC | 30.40 ± 0.463 |
| AP3-KC | AP3-KC | 0.11 ± 0.032 |
| PI-KC | PI-KC | 0.13 ± 0.024 |
| OsMADS16-K | OsMADS4-KC | 80.67 ± 5.630 |
| OsMADS16-K | PI-KC | 53.26 ± 4.342 |
| PI-KC | OsMADS16-KC | 69.58 ± 5.520 |
| OsMADS16-K | AP3-KC | 0.11 ± 0.014 |
| AP3-KC | OsMADS16-KC | 0.07 ± 0.022 |
| OsMADS4-KC | AP3-KC | 0.12 ± 0.012 |
| AP3-KC | OsMADS4-KC | 0.10 ± 0.027 |
| OsMADS4-KC | PI-KC | 0.09 ± 0.021 |
| PI-KC | OsMADS4-KC | 0.07 ± 0.039 |
| AP3-KC | - | 0.09 ± 0.012 |
| PI-KC | - | 0.11 ± 0.022 |
| OsMADS16-K | - | 0.09 ± 0.032 |
| OsMADS4-KC | - | 0.12 ± 0.042 |
| - | AP3-KC | 0.05 ± 0.012 |
| - | PI-KC | 0.07 ± 0.013 |
| - | OsMADS16-KC | 0.08 ± 0.011 |
| - | OsMADS4-KC | 0.08 ± 0.013 |

[a]β-galactosidase activity unit = 1000 x $OD_{420}$/[$OD_{600}$ x reaction time (min) x volume of culture (mL)].

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 the 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.

*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 ± 0.012 |
| PI-KC | 0.11 ± 0.022 |
| OsMADS4-KC | 0.12 ± 0.042 |
| OsMADS16-KC | 72.98 ± 7.188 |
| OsMADS16-K | 0.09 ± 0.032 |
| OsMADS16-$C_{155\text{-}223}$ | 65.12 ± 4.070 |
| OsMADS16-$C_{181\text{-}223}$ | 0.12 ± 0.023 |
| OsMADS16-$C_{201\text{-}223}$ | 0.07 ± 0.039 |
| OsMADS16-$C_{155\text{-}180}$ | 0.25 ± 0.008 |
| OsMADS16-$C_{155\text{-}200}$ | 145.61 ± 7.673 |

[a]β-galactosidase activity unit = 1000 x $OD_{420}$/[$OD_{600}$ x reaction time (min) x volume of culture (mL)].

*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 155 and 200 amino acid residues plays an important role in transcription activation ability of the OsMADS4 protein.

*Isolation of OsMADS6*

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 [3]. 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; genbank accession No. U78782). The MADS box domain of the cDNA clone is located between the 2nd and 57th amino acids of the protein (Figure 4A). 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 [27]. The C-terminal region of OsMADS6 has two short motifs (EPTLQIG and AENNFMLGWVL) that are partially conserved in *ZAG3*, *ZAG5*, *AGL2*, *AGL4*, and *AGL9* (Figure 4A). Based on amino acid sequence similarity of the entire coding region, *OsMADS6* can be grouped into the *AGL2* family [27] which includes *AGL2* and *AGL4* of *Arabidopsis* [20], *ZAG3* and *ZAG5* of maize [23], *FBP2* of petunia [1], *TM5* of tomato [26], and *OsMADS1*, *OsMADS5*, *OsMADS7*, and *OsMADS8* of rice [3, 14, 15]. Among these genes, *OsMADS6* was the most homologous to *ZAG3* (84%) and *ZAG5* (82%).

*Identification of MADS box proteins that interact with OsMADS6*

To identify proteins that interact with OsMADS6, yeast two-hybrid screening was conducted. 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 *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. Therefore, we made another molecule, which was identical to pBD/OsMADS6-KC except that most of the C-

**A**

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MGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYEFGSA   OsMADS6
************************************************************   ZAG3
*************************************************G**********   ZAG5
******M****************************************************V   AGL6

GITKTLERYQHCCYNAQDSNN-ALSETQSWYHEMSKLKAKFEALQRTQRHLLGEDLGPLS
********************G-********Q*****R****************E******
*V*******************S****S****Q*****R**********************
**ES*I***NR-***CSL***KPEET****CQ*VT***S*Y*S*V**N*N*******EMG

VKELQQLEKQLECALSQARQRKTQLMMEQVEELRRKERQLGEINRQLKHKLEVEGSTSNY
**************************************H***M*********A**-C***
************************V**********T**H***M*********A**-C***
*****A**R***A**TAT******V***EM*D**K******D**K***I*F*T**--HAF

RAMQQAS---WAQGAVVEN-GAAYVQPPP--HSAAMDSEPTLQIGYP--HQFVPAEAN-T
*TL*H*A-WPAPGSTM**HD**T*HVH*TTAQ*V***C*********PH***L*S**ANN
TTL*H*ACWPAPG*TI**HD**T*QVH**-A**V***C*********-H***P*P**VNN
KTF*DLWANSA*SV*GDP*-NSEFPVE*SHPNVLDCNT**F****FQ-Q*YY*QG*GS-S

IQRSTAPAGAENNFMLGWVL
*P**--*P*G**********
*P**--A*TG**********
VSK*--NVAG*T**VQ****
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**B**

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MGRGKVQLKRIENKINRQVTFSKRRSGLLKKANEISVLCDAEVALIIFSTKGKLYEYATD   OsMADS14
*************************N******H***********V*V**P**********   ZAP1

SCMDKILERYERYSYAEKVLISAESDTQGNWCHEYRKLKAKVETIQKCQKHLMGEDLESL
*R****************A******ESE*************I******H***********

NLKELQQLEQQLENSLKHIRSRKSQLMLESINELQRKEKSLQEENKVLQKELVEKQ----
*P**********DS**********H**A***S***K**R*******A*****A*R*KAVA

---KVQKQQVQWDQT---QPQTSSSSSSFMMR---EALP-TTNISNYPAAAGERIEDVAA
SRQQQ*Q*******QTHA*A************QDQQG**PPH**CFP*LTM*D*G*EL**

G-------------QPQHERIGLPPWMLSHING
AAAAQQQQPLPGQA***LRIA*********L*A
```

*Figure 4.* The deduced amino acid sequence comparisons of MADS 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, which were introduced to maximize alignments.

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 histidine, 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 x $10^6$ transformants was screened for their ability to grow on a medium lacking histidine. This initial screening identified 59 colonies, which were subsequently tested for activation of the *LacZ* gene. These experiments resulted in identification of 45 colonies that activated both *HIS3* and *LacZ*. Plasmid DNAs were prepared from these colonies and retransferred 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 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 proteins (Table 4). The remaining plasmid had some

*Table 4.* The MADS proteins isolated by yeast two-hybrid screening using OsMADS6 as a bait.

| Gene name | No. of isolated clone(s) | Homologous gene | Reference |
|---|---|---|---|
| OsMADS1 | 6 | AGL2 | Chung et al., 1994 |
| OsMADS5 | 2 | AGL2 | Kang and An, 1997 |
| OsMADS7 | 3 | AGL2 | Kang et al., 1997 |
| OsMADS14 | 12 | ZAP1 | This study |
| OsMADS15 | 13 | ZAP1 | This study |
| OsMADS17 | 1 | ZAG3 | This study |
| OsMADS18 | 1 | ZAP1 | This study |

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 proteins; six clones belong to *OsMADS1* [3], two to *OsMADS5* [14], and three to *OsMADS7* [15]. The remaining plasmids encode for MADS box proteins, which were not previously reported. Twelve of these were partial clones of an identical MADS gene, although the 5′ ends were different from each other (Figure 5). This gene was designated *OsMADS14*. Thirteen clones encoded for an identical protein of another MADS 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, designated *OsMADS17*, showed a high similarity with *ZAG3*, and the last clone, designated *OsMADS18*, was the most homologous to *ZAP1*.

*Isolation of the OsMADS14 cDNA clone containing an entire open reading frame*

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 (Figure 5). 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 open reading frame was generated by connecting the 5′ region to the cDNA clone obtained from the two-hybrid screening. It contains a 287 bp 5′ untranslated region and an open reading frame of 246 amino acid residues (calculated $M_r$ = 28,500; genbank accession No. AF058697).

The 5′ UTR of *OsMADS14* cDNA contains 10 repeats of the GGA sequence (Figure 5), and such repeat sequences were previously observed from other rice MADS-box genes [3, 14, 15]. 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 (Figure 4B and Figure 5). The K box domain, a region considered to participate in protein-protein interaction, is also present in between amino acid residues 91 to 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 genes in the rice genome [3]. Therefore, it was necessary to identify the region that does not cross hybridize with other MADS genes by genomic DNA blot analyses. It was observed that the 300 bp *Pst*I-*Eco*RI fragment which is located at the C-terminal region of *OsMADS6* hybridized to a single DNA fragment (Figure 6A, right). Likewise, the 630 bp *Xho*I fragment of *OsMADS14* was shown to be a gene specific region. In genomic DNA analysis of *OsMADS14*, three *Pst*I fragments were hybridized with the probe (Figure 6A, left). This was due to the presence of two *Pst*I sites in the region that was 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 (Figure 6B). However, the transcript was not detectable in stamens, paleas/lemmas, and vegetative organs. Spatial expression pattern of *OsMADS14* was different from that of *OsMADS6*. Transcripts of this clone were detectable primarily in sterile lemmas, and also weakly in paleas/lemmas, stamens, and carpels (Figure 6B). However, the *OsMADS14* transcripts were not detected in lodicules and vegetative organs. During flower development, the *OsMADS6* and *OsMADS14* genes were expressed at the early stage of the flower development, and their expressions were extended into later stages of flower development (Figure 6B).

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 CT CGA TCG ATC CAT CCA TCG ATC GAT CGG TCC CCC CCA CCG CGC GCG ACG CAT TCC GCC     59
GCC GTC TCG CCG TGT CCA CGT GAT GGG GGC CGG 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 AAG AAC AGG AGG AAG ATG GGG CGG GGC    299
                                                                 M   G   R   G      4

AAG GTG CAG CTG AAG CGG ATC GAG AAC AAG ATC AAC CGG 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 TCG 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
                1→  1→                      1→      1→
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
 2→                                             1→
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
                                    2→      1→
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
                    1→          1→
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 CAG AAA GAA CTG GTG GAG AAG CAG AAA GTC CAG AAG CAA CAA GTG CAA    839
 N   K   V   L   Q   K   E   L   V   E   K   Q   K   V   Q   K   Q   Q   V   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 GCA 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 AGC ACC GTC TTC CTC CCA CCA GTA ATT TAC CCA TGT AAT ATA TGC GAG CAG CAT   1259
GAA CAA ATT TTC CCG TTT CCA ACC ATC TCC ATT GGT GTC ATG TGT GAC TTA AAT AGC GAA   1319
ATT TCA GCA TTG TGC ATA GTG TGA TTA CTG TAA GAT                                   1355
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*Figure 5.* 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 ten repeats of the GGA sequence in the 5′ untranslated region are indicated in a 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 *Xho*I site used for generation of the gene-specific probe is indicated in bold type. Genbank accession No. is AF058697.

*Interaction between OsMADS6 and other MADS proteins*

The yeast two-hybrid screening resulted in identification of seven types of MADS proteins that interact with OsMADS6. In order to confirm the results, we investigated the protein-protein interaction between OsMADS6 and other rice MADS 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 a yeast strain YRG-2 containing the binding domain

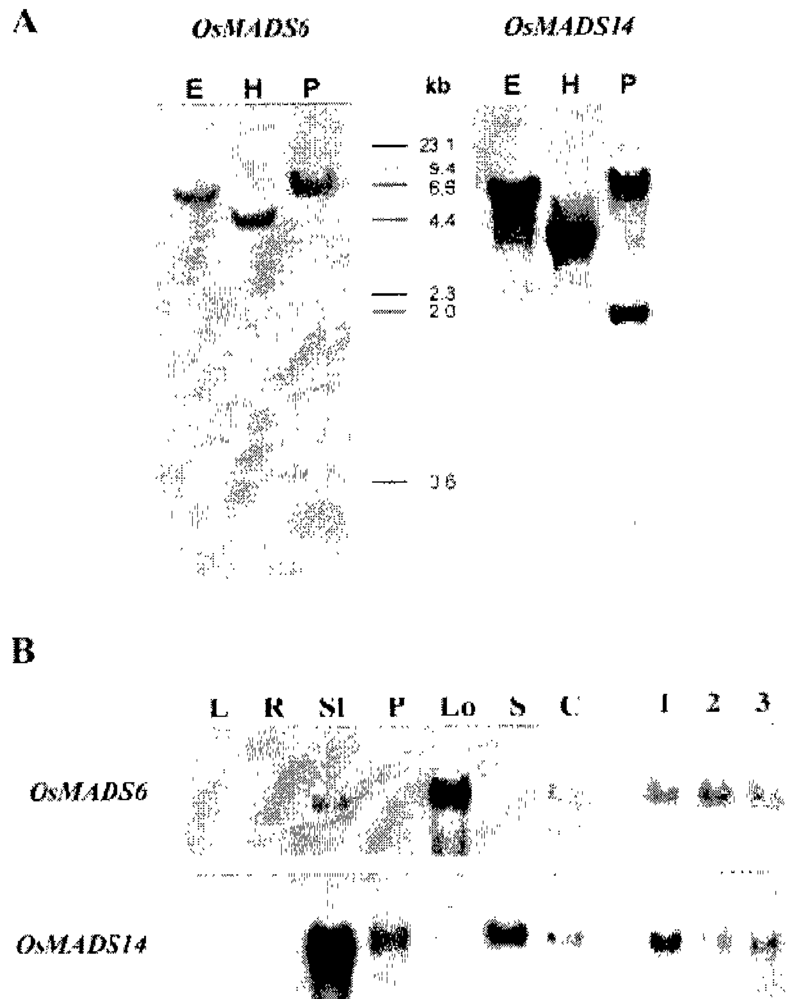


*Figure 6.* Genomic DNA blot analysis and RNA blot analysis of *OsMADS6* and *OsMADS14*. A, Southern blot analysis of *OsMADS6* and *OsMADS14*. The rice genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H), or *Pst*I (P). The numbers indicate the size, in kb, of the DNA markers. B, RNA blot analysis of *OsMADS6* and *OsMADS14*. Ethidium bromide staining of 25S and 17S rRNAs demonstrated equal amounts of RNA loading (data not shown). L, leaves; R, roots; Sl, sterile lemmas; P, paleas/lemmas; Lo, lodicules; S, stamens; C, carpels; 1, young flowers at the panicle size 1 to 5 cm; 2, flowers at early vacuolated pollen stage; 3, flowers at late vacuolated pollen stage.

plasmid, pBD/OsMADS6-KC14. The colonies that grew on a medium lacking leucine and tryptophan were examined for β-galactosidase activity. The results in Table 5 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).

In order 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 OsMAS4 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 containing 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 MADS box proteins,

*Table 5.* Quantitative assay of protein-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 = 1000 x $A_{420}/[A_{600}$ x reaction time (min) x volume of culture (mL)].
[b]GAL4 DNA binding domain fusion protein.
[c]pBDGAL4 is used as a negative control.

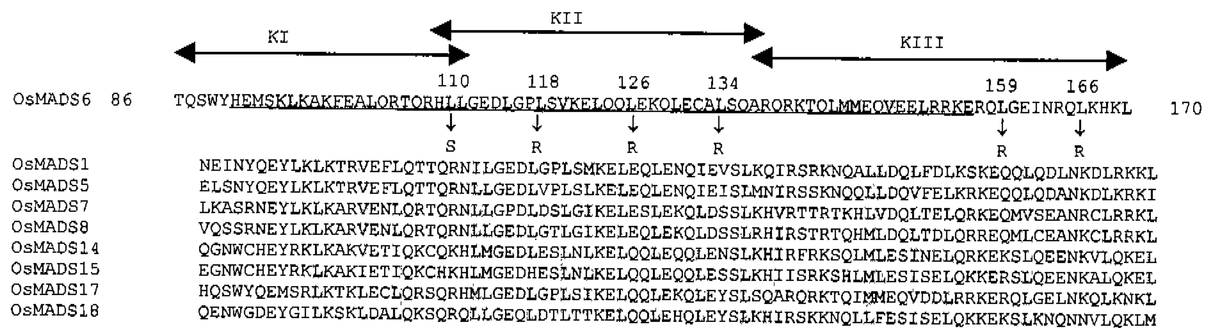


*Figure 7.* The amino acid sequence alignment of the region containing the K domain of OsMADS6 with those of OsMADS1, 5, 7, 8, 14, 15, 17, and 18. The region of OsMADS6 was divided into three regions; KI region (from 86[th] to 110[th] aa), KII region (from 109[th] to 137[th] aa), and KIII region (from 138[th] to 170[th] aa). The entire K domain of OsMADS6 is underlined. The replaced amino acids are indicated below each leucine with arrows. The conserved hydrophobic residues, such as leucine, isoleucine, valine, and methionine, are shaded. The numbers indicate the position of mutagenized leucines, and the first and last amino acids of the K region elucidated in this study.

OsMADS4 and OsMADS3. It also failed to interact to OsMADS6 itself.

*Identification of the protein-protein interaction motif*

The motif responsible for the protein-protein interaction between OsMADS6 and OsMADS14 was investigated using the yeast two-hybrid system. The MADS box domain and 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 leucine and tryptophan were examined for activation of the *LacZ* gene by a β-galactosidase activity analysis (Table 6). 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 171[st] to 250[th] aa of Os-

Table 6. Investigation of the motif responsible for protein-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 = 1000 x $A_{420}$/[$A_{600}$ x reaction time (min) x volume of culture (mL)].

MADS6 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 6). 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 five- or twenty-fold (Table 6), 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-protein interaction, the KC14 region of OsMADS6 was divided into three regions; KI (86$^{th}$ to 110$^{th}$ aa), KII (109$^{th}$ to 137$^{th}$ aa), and KIII (138$^{th}$ to 170$^{th}$ aa) (Figure 7). 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-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 a fact that pAD/OsMADS6-KII did not activate the *LacZ* gene in the presence of pBD/OsMADS14-KC14 (Table 6). Each activation domain or binding domain plasmid used in these experiments did not activate the *LacZ* gene by itself (data not shown).

### *Identification of the amino acid residues responsible for the protein-protein interaction*

Because it was identified that the KII and KIII regions containing the 109$^{th}$ to 170$^{th}$ aa of OsMADS6

*Table 7.* 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/OsMADS14-KC | pBD/OsMADS6-KII | 10.52 ± 2.460 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KIIS$_{110}$R$_{118}$ | 0.17 ± 0.016 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KIIR$_{126}$R$_{134}$ | 0.05 ± 0.015 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KII+KIII | 50.63 ± 1.983 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KIIS$_{110}$R$_{118}$+KIII | 29.33 ± 2.660 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KIIS$_{110}$R$_{118}$+KIIIR$_{159}$R$_{166}$ | 0.07 ± 0.012 |
| pAD/OsMADS14-KC | pBD/OsMADS6-KII+KIIIR$_{159}$R$_{166}$ | 0.07 ± 0.016 |
| pAD/OsMADS6-KII | pBD/OsMADS14-KC14 | 0.09 ± 0.027 |
| pAD/OsMADS6-KIIS$_{110}$R$_{118}$ | pBD/OsMADS14-KC14 | 0.08 ± 0.032 |
| pAD/OsMADS6-KIIR$_{126}$R$_{134}$ | pBD/OsMADS14-KC14 | 0.08 ± 0.016 |
| pAD/OsMADS6-KII+KIII | pBD/OsMADS14-KC14 | 13.91 ± 0.905 |
| pAD/OsMADS6-KIIS$_{110}$R$_{118}$+KIII | pBD/OsMADS14-KC14 | 3.11 ± 0.247 |
| pAD/OsMADS6-KIIS$_{110}$R$_{118}$+KIIIR$_{159}$R$_{166}$ | pBD/OsMADS14-KC14 | 0.09 ± 0.007 |
| pAD/OsMADS6-KII+KIIIR$_{159}$R$_{166}$ | pBD/OsMADS14-KC14 | 0.09 ± 0.013 |

[a]β-galactosidase activity unit = 1000 x $A_{420}/[A_{600}$ x reaction time (min) x volume of culture (mL)].

play an important role in the protein-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 leucine residues (Figure 7). It has been previously suggested that such hydrophobic repeats may be involved in protein-protein interactions [20, 25, 32]. Therefore, we made the following five mutant fragments: OsMADS6-KIIS$_{110}$R$_{118}$: replacement of 110$^{th}$ leucine residue with serine and 118$^{th}$ leucine with arginine in the KII fragment; OsMADS6-KIIR$_{126}$R$_{134}$: replacement of 126$^{th}$ and 134$^{th}$ leucines with arginines in the KII fragment; OsMADS6-KIIS$_{110}$R$_{118}$+KIII: replacement of 110$^{th}$ leucine with serine and 118$^{th}$ leucine with arginine in the KII+KIII fragment; OsMADS6-KII+KIIIR$_{159}$R$_{166}$: replacement of 159$^{th}$ and 166$^{th}$ leucines with arginines in the KII+KIII fragment; and OsMADS6-KIIS$_{110}$R$_{118}$+KIIIR$_{159}$R$_{166}$: replacement of 110$^{th}$ leucine with serine, and 118$^{th}$, 159$^{th}$ and 166$^{th}$ leucines with arginines 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 7 show that mutations in leucines at the 110$^{th}$ and 118$^{th}$ residues diminished the interacting ability of the KII fragment. Similarly, mutations of the leucines at the 126$^{th}$ and 134$^{th}$ residues also significantly affected the activity. These results suggest that the four periodical leucine residues in the KII region are necessary for interaction between the K box regions. When the mutations at the 110$^{th}$ and 118$^{th}$ residues were introduced into the KII+KIII fragment, the enzyme activity was reduced, but still retained a significant level of the activity. However, when mutations were introduced into both KII and KIII regions by replacing four leucines at 110$^{th}$, 118$^{th}$, 159$^{th}$, and 166$^{th}$ residues, the activity was almost completely diminished. These results together with the results in Table III indicate that the KIII region alone is not sufficient for protein interaction, but able to enhance the interaction between MADS proteins. When the mutant fragments were used in 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 7). Taken together, these results suggest that the leucine residues in the KII region are important for the protein interaction and the leucines in the KIII region are involved in enhancing the interaction.

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