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Two rice MADS domain proteins interact with *OsMADS1*

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Abstract

OsMADS1 is a MADS box gene controlling flower development in rice. In order to learn more about the function of *OsMADS1*, we searched for cellular proteins interacting with *OsMADS1* employing the yeast two-hybrid system. Two novel proteins with MADS domains, which were named *OsMADS14* and *OsMADS15*, were isolated from a rice cDNA library. *OsMADS14* and *-15* are highly homologous to the maize MADS box gene *ZAP1* which is an orthologue of the floral homeotic gene *APETALA1 (AP1)*. Interactions among the three MADS domain proteins were confirmed by *in vitro* experiments using GST-fused *OsMADS1* expressed in *Escherichia coli* and *in vitro* translated proteins of *OsMADS14* and *-15*. We determined which domains in *OsMADS1, -14*, and *-15* were required for protein-protein interaction employing the two-hybrid system and pull-down experiments. While the K domain was essential for protein-protein interaction, a region preceded by the K domain augmented this interaction. Interestingly, the C-terminal region of *OsMADS1* functioned as a transcriptional activation domain in yeast and mammalian cells, while, on the other hand, the C domains of *OsMADS14* and *-15* exhibited only very weak transcriptional activator functionality, if any at all.

Abbreviations: ADH1, alcohol dehydrogenase 1; 5′-RACE, rapid amplification of 5′-cDNA ends; RRL, rabbit reticulocyte lysate; CAT, chloramphenicol *O*-acetyltransferase

Introduction

Dramatic changes occur at the transition from the vegetative to the reproductive phase during plant development. Flower development is triggered by both endogenous signals, such as age, and environmental signals, such as day length. Upon initiation of the reproductive phase in *Arabidopsis thaliana*, the development of the floral meristem is initiated by floral meristem identity genes, such as *LEAFY (LFY), APETALA1 (AP1), APETALA2 (AP2)*, and *CAULIFLOWER (CAL)*. At a later phase, the fate of the floral organ primordia is specified by homeotic genes (for a review see Weigel and Meyerowitz, 1994) such as *AP1, AGAMOUS (AG), PISTILATA (PI)*, and *APETALA3 (AP3)*. These homeotic genes belong to the MADS domain protein gene family. The encoding MADS box genes contain a highly conserved stretch of 56 amino acids, which was first discovered as a conserved motif among the genes *MCM1, AGAMOUS, DEFICIENS*, and serum responsive factor (Sommer and Schwarz-Sommer *et al.*, 1990). MADS box genes encode transcription factors that participate in signal transduction and developmental control. They exist in plants, animals, yeast, and fungi.

A number of MADS box genes have been identified by molecular biological techniques that exploit sequence similarities. At least 28 MADS box genes of *Arabidopsis* have been isolated (Riechmann and Meyerowitz, 1996). Some MADS box genes are expressed in a temporally and/or spatially regulated manner, and they appear to be involved in controlling floral organ initiation and development. For example, *AGL1* and *AGL4* are expressed very early in flower devel-

opment after the floral meristem has emerged from the inflorescence meristem but before any of the organ primordia have emerged (Flanagan and Ma, 1994; Savidge *et al.*, 1995). This suggests that *AGL2* and *AGL4* may play important roles in the intermediate step between inflorescence initiation and floral organ initiation.

The MADS box is required for DNA binding and protein dimerization (Hayes *et al.*, 1988; Norman *et al.*, 1988). Plant MADS box proteins contain a second domain, a moderately conserved region, which is not present in the MADS proteins of other organisms. This domain is called K domain for its similarity to the coiled-coil domain in keratin. It has been demonstrated that the K domain is required for interactions among the plant MADS domain proteins (Ma *et al.*, 1991; Davies *et al.*, 1996; Fan *et al.*, 1997). For example, *AG* was seen to interact with *AGL2, AGL4, AGL6*, and *AGL9* in experiments using the yeast two-hybrid system (Fan *et al.*, 1997). The K domain was indispensable in this protein-protein interaction. This indicates that MADS domain proteins cooperate with other MADS proteins by K domain-mediated interaction to carry out their functions. The intervening region between MADS and K is called the I domain. In the case of *AP3* and *PI*, both the MADS and the I domains are required for nuclear localization of the proteins (McGonigle *et al.*, 1996). The C-terminal regions of the MADS box genes are highly diverse. Recently we have shown, by using yeast and mammalian gene expression systems, that the C domain of *AP1* of *A. thaliana* and its homologues functions as a transcriptional activation domain (Cho *et al.*, 1999).

Although many more pioneering investigations into the functions of MADS box genes have been pursued in the two dicots *A. thaliana* and *A. majus*, MADS box genes have also been identified in monocots including maize, sorghum, orchid, and rice (Lu *et al.*, 1993; Schmidt *et al.*, 1993; Montag *et al.*, 1995; Kang *et al.*, 1995, 1997; Greco *et al.*, 1997; Kang and An, 1997). Among them, *OsMADS1*, a rice homologue of *AGL2* (Chung *et al.*, 1994), seems to play a key role in flower induction. The *OsMADS1* gene is actively expressed at the young inflorescence stage, and the expression continues into the early and vacuolated pollen stage (Chung *et al.*, 1994). The gene is initially expressed uniformly in young flower primordia but becomes more localized in the pela, lemma, and ovary at later developmental stages (Chung *et al.*, 1994). Vegetative tissues do not show any expression of the gene. Ectopic expression of the *OsMADS1* in homologous and heterologous plants results in early flowering. Therefore it is likely that the rice *OsMADS1* product regulates expression of genes involved in the induction of flowers.

In order to uncover the role of *OsMADS1* in flower development, we looked at the various functional domains of *OsMADS1*. We found that the K domain is essential for protein-protein interaction of *OsMADS1* with other MADS domain proteins. The C domain of *OsMADS1*, like that of *AP1*, functioned as a transcriptional activation domain in yeast and mammalian cells, systems that have been widely used for testing transcriptional activator functions. In addition, proteins interacting with *OsMADS1* have been investigated with the yeast two-hybrid system (Fields and Song, 1989). We isolated two novel MADS box genes, named *OsMADS14* and *-15*, from a rice cDNA library generated from young rice flowers. The protein-protein interaction between *OsMADS1* and these proteins was confirmed by *in vitro* pull-down assay. The K domains of all three proteins were required for protein-protein interaction, and a segment following the K domains augmented the interaction. The C domains of the two novel proteins showed very weak, if any, transcriptional activation function in yeast and a mammalian cell line. Possible implications of the interactions between *OsMADS1, OsMADS14*, and *OsMADS15* are discussed.

Materials and methods

Plasmid construction

Cloning of plasmids was carried out by standard methods (Sambrook *et al.*, 1989). For the yeast two-hybrid system, plasmids pGBT9 and pGAD424 were used as sources of GAL4 DNA-binding domain and GAL4 activation domain, respectively (Clontech). GAL4-fusion genes are under the control of the yeast ADH1 promoter. To generate pGBT9/*OsMADS1*, consisting of a full-length *OsMADS1* gene in pGBT9, an *Eco*RI fragment of plasmid pSK(-)/*OsMADS1* (Chung *et al.*, 1994) was inserted into the *Eco*RI site of vector pGBT9. Deletion mutants of *OsMADS1* were made from pSK/*OsMADS1*. The series of DNA inserts for *OsMADS1*-KC (88–257), *OsMADS1*-ΔKC (109–257), and *OsMADS1*-CII (160–257) were generated by treating pSK/*OsMADS1* with *Ase*I-*Eco*RI-Klenow, *Eco*RI-Klenow-*Ssp*I, and *Bgl*II-*Eco*RI-Klenow, respectively. The vectors pGBT9

and pGAD424 were treated with *Sma*I-*Bam*HI-Klenow and with *Sal*I-Klenow-*Sma*I, respectively. The insert DNA for clone pGBT9/*OsMADS1*-ΔC (1–218) was prepared by polymerase chain reaction (PCR) with pSK/*OsMADS1* as template and 5′-CGCGGATCCAGATGGGGAGGGGGAAGG-3′, and 5′-TGCCTGCAGGGAATGGTCACCC-3′ as primers. Plasmid pGBT9 and the PCR products were digested with *Bam*HI-*Pst*I, and then ligated to generate plasmid pGBT9/*OsMADS1*-ΔC (1–218). pGBT9/*OsMADS1*-KΔC (88–218) was constructed by inserting a DNA fragment of pGBT9/*OsMADS1*-ΔC (1–218) generated by *Ase*I-Klenow-*Psi*I into the pGBT9 vector treated with *Sma*I-*Psi*I. To generate DNA fragments for plasmids pGAD424/*OsMADS14*-KC (93–246), pGAD424/*OsMADS14*-K (93–158), pGAD424/*Os MADS14*-K′ (93–174), pGAD424/*OsMADS14*-C (159–246), pGAD424/*OsMADS15*-KC (93–268), pGAD424/*OsMADS15*-K (93–158), pGAD424/ *OsMADS15*-K′ (93–174), and pGAD424/*OsMADS15*-C (159–268), PCRs were performed using as templates the DNA of *OsMADS14* and *OsMADS15* and proper pairs of ten primers: primer 1 (*15*-K), 5′-CCCAGAATTCCATGAATACAGGAAAC-3′; primer 2 (*15*-anti-K), 5′-CACGCGGATCCACTCTCCTTTTT CTGCAGC-3′; primer 3 (*15*-C), 5′-CCCAGAATTCA GGTCACTGCAGGAGG-3′; primer 4 (*15*-anti-C), 5′-CACGCGGATCCTTAAGCATTGAGTGGCTC-3′; primer 5 (*15*-anti-K′), 5′-CACGCGGATCCTTACTC CACCAGTTCCTTC-3′; primer 6 (*14*-K), 5′-CCCA GAATTCCACGAATATAGGAAAC-3′; primer 7 (*14*-anti-K), 5′-CACGCGGATCCACTTTCCTTCCGTTG AAGC-3′; primer 8 (*14*-C), 5′-CCCAGAATTCAAG TCACTGCAGGAGG-3′; primer 9 (*14*-anti-C), 5′-CACGCGGATCCTTAGCCGTTGATGTGGCTC-3′; and primer 10 (*14*-anti-K′), 5′-CACGCGGATCCTTAT TTCTGCTTCTCCACC-3′. PCR product and vector DNA (pGAD424) were co-digested with *Eco*RI and *Bam*HI and the DNA fragments ligated to generate the yeast vectors used in the two-hybrid analyses. The same set of PCR products was also used to construct the plasmids for *in vitro* translation: pTM1/*OsMADS15*-KC (93–268), pTM1/*OsMADS15*-K (93–158), pTM1/*OsMADS15*-K′ (93–174), pTM1/*OsMADS15*-C (159–268), pTM1/*OsMADS14*-KC (93–246), pTM1/*OsMADS14*-K (93–158), pTM1/*OsMADS 14*-K′ (93–174), and pTM1/*OsMADS14*-C (159–246). For this purpose, vector pTM1, which contains T7 promoter followed by EMCV IRES, was used. pTM1 treated with *Sma*I and *Bam*HI was ligated to the PCR products treated with *Eco*RI-Klenow-*Bam*HI. Plasmid pSG424 contains the GAL4 DNA-binding domain (amino acids 1–147) under the control of the SV40 promoter. To construct pSG424/*OsMADS1*-ΔKC, the vector pSG424 was treated with *Bam*HI-Klenow and ligated to pSK(–)/*OsMADS1* treated with *Eco*RI-Klenow-*Ssp*I. pSG424/*OsMADS14*-KC and pSG424/*OsMADS15*-KC were constructed by ligating vector pSG424 treated with *Sac*I-T4 polymerase-*Eco*RI to the respective PCR products treated with *Bam*HI-Klenow-*Eco*RI. To construct pGEX/*OsMADS1*-KΔC (88–218), a ligation was performed between vector pGEX-KG, which contains GST protein controlled by the Tac promoter, treated with *Hind*III-Klenow and the insert of pGBT9/*OsMADS1*-KΔC treated with *Ase*I-*Dra*I-Klenow.

Yeast two-hybrid screening

An expression cDNA library for yeast two-hybrid screening was generated from mRNA isolated from young rice flowers with 2–5 cm panicles using the Hybrid-ZAP vector following the manufacturer's suggestions (Stratagene). The average insert size was 0.9 kb. The cDNA sequences of clones scoring positive in the yeast two-hybrid screening were determined with the dideoxynucleotide chain termination sequencing method (Sanger *et al.*, 1977) using oligonucleotide primers annealing to the GAL4 activation domain. The DNA sequences were analyzed with the BLAST program using the database of the National Center for Biotechnology Information.

The yeast strain HF7C [*MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-901, leu2-3, 112, gal4-542, gal80-538,* cyh^r2, *LYS2::GAL1*$_{uas}$-*GAL1*$_{tata}$-*HIS3*, *URA3::GAL4*$_{17mers}$(*X3*)-*CyCl*$_{tata}$-*lacZ*] was used for the two-hybrid screening. The *lys2* gene is non-functional. The *trp1, leu2, his3, gal4*, and *gal80* mutations are all deletions. The GAL1 upstream activating sequence (UAS) and the three tandem copies of the GAL4 17-mer consensus sequence [$GAL_{17mers}(X3)$] respond to either the GAL4 transcriptional activator or a potential transcriptional activator fused to the Gal4 DNA-binding domain. HF7C encodes *HIS3* and β-galactosidase as reporters and has *trp1, leu2*, and cyh^r2 as marker genes for transformant selection (Feilotter *et al.*, 1994).

Saccharomyces cerevisiae HF7C was grown in YPD (1% yeast extract, 2% peptone, 2% D-glucose, 1.5% agar for plates) or synthetic minimal medium (0.67% yeast nitrogen base, 2% D-glucose, (10×)

amino acids dropout solution deficient in tryptophan or histidine, and 1.5% agar for plates). HF7C was transformed with pGBT9-*OsMADS1*-ΔC (1–218) or the cDNA library by the lithium-acetate method (Gietz *et al.*, 1992), and transformants were selected on the synthetic minimal medium (SD) depleted of leucine, histidine, and tryptophan (SD, leu^-, trp^-, his^-). Yeast colonies grown on SD, leu^-, trp^-, his^- plates were transferred to a filter (Whatman No. 1). The filter was soaked with liquid nitrogen for 30 s and then incubated in Z-buffer (60 mM Na_2HPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$) containing 0.82 mM X-gal. Colonies developing a blue color were picked and cultivated again on SD, leu^-, trp^-, his^- plates. Yeast plasmids containing prey genes were isolated and then retested in the two-hybrid system using the same bait and pGBT9 as a negative control to remove false-positives. The sequences of the true positive clones were analyzed using the Sanger (1977) method. An *OsMADS15* cDNA clone containing the entire open reading frame was obtained by 5′-RACE using the Marathon-Ready kit (Clontech).

In vitro *pull-down assay*

The GST protein and the GST/*OsMADS1*-KΔC (88–218) fusion protein from the plasmids pGEX-KG and pGEX/*OsMADS1*-KΔC (88–218), respectively, were expressed in *E. coli* strain BL21 (DE3). The proteins were solubilized in lysis buffer (20 mM Na-Pi, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol containing 100 μg/ml lysozyme) and bound to glutathione Sepharose 4B resin (Pharmacia) in 1 ml of lysis buffer for 1 h at 4 °C. The resin was then washed three times with lysis buffer. [^{35}S]-labeled proteins were synthesized in a rabbit reticulocyte lysate (RRL) *in vitro* translation system as described by the manufacturer (Promega). The *in vitro* translation mixtures were incubated with resin in binding buffer (20 mM Na-Pi, 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol containing 100 μg/ml lysozyme) for 2 h at 4 °C and washed three times with 1 ml binding buffer. The resin-bound proteins were resolved on a 15% SDS-polyacrylamide gel.

Transfection of mammalian cells

Transcriptional activator function of *OsMADS1, -14*, and *-15* in mammalian cells was tested using COS-7 cells. The COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% bovine calf serum. Transfections were carried out by the electroporation method (Tatsuka *et al.*, 1988). Briefly, DNAs (3 μg of the pSG424 expression vector or its derivatives, 3 μg of pG5E1b-CAT reporter plasmid, and 0.1 μg of control plasmid pGL2 expressing luciferase, Stratagene) were co-transfected into COS-7 cells using the Bio-Rad Gene Pulser apparatus at 960 μF and 0.25 kV in 1 ml DMEM without serum. After electroporation, the cells were maintained in DMEM supplemented with 10% bovine calf serum and 5% fetal bovine serum on 100 mm dishes. At 48 h after transfection, the cells were harvested and resuspended in 200 μl of phosphate-buffered saline (PBS) containing 5 mM EDTA.

Chloramphenicol acetyl transferase assay

Chloramphenicol acetyl transferase (CAT) activity in COS-7 cells was measured by the method described by Neumann *et al.* (1987). Cell extracts were prepared by four cycles of freeze-thawing in 200 μl of PBS containing 5 mM EDTA. The supernatant of a 5-min centrifugation in an Eppendorf microcentrifuge at 4 °C was used for CAT assay. CAT activity was measured in 231 μl of a reaction mixture containing 1 μl of [^{3}H]-acetyl CoA (250 μCi/ml; Amersham), 5 μl of unlabeled acetyl CoA (1 mg/ml in H_2O), and 25 μl of 10 mM chloramphenicol. The reaction mixture was gently overlaid onto 3 ml of Econofluor-2 (Packard) and then incubated at 37 °C. After 30 and 60 min, the amount of [^{3}H]-acetyl CoA transferred to chloramphenicol was measured in a liquid scintillation counter (1600 CA, TRI-CARB). Plasmids pSG147-VP16 and pSG424 were used as positive and negative controls, respectively. CAT activity was normalized to the luciferase activity obtained from the pGL2 control vector in order to correct for the transfection efficiency in each experiment.

β-galactosidase assay

Mid to 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 the substrate (ONPG assay). Units of activity were calculated with the formula: $1000 \times A_{420}/(A_{600} \times$ assay time in min $\times$ assay volume in ml.

Results

The C-terminal region of OsMADS1 *contains a transcriptional activation domain*

Recently we have shown that the C region of *AP1*, a plant MADS domain protein, contains transcriptional activation domains that function synergistically (Cho *et al.*, 1999). This implies that some of the MADS domain proteins function as transcriptional activators. We investigated whether *OsMADS1* contained transcriptional activation domain(s) using yeast and mammalian gene expression systems. The full-length *OsMADS1* clone showed transcriptional activator function as indicated by the viability of yeast cells on the SD, trp^{-}, his^{-} medium (Figure 1B, (a)). Deletion mutants of *OsMADS1* still containing the C domain (Figure 1A, (c), (d), and (e)) all exhibited transcriptional activator functionality (Figure 1B, (c), (d), and (e)). On the other hand, mutants with deletions of the C-terminal end of the C domain (amino acids 218–257; Figure 1A, (b) and (f)) had abolished the transcriptional activator function as indicated by the mortality of the yeast transformants on SD, trp^{-}, his^{-} medium (Figure 1B, (b) and (f)). This suggests that the C domain of *OsMADS1* functions as a transcriptional activation domain in yeast cells. The transcriptional activator function of *OsMADS1* was confirmed in mammalian cells (see below).

Yeast two-hybrid screening for proteins interacting with OsMADS1

To identify proteins interacting with the *OsMADS1* protein, the yeast two-hybrid system was employed. Full-length *OsMADS1* is not suitable for the yeast two-hybrid system, since the C-terminal end of *OsMADS1* contains a transcriptional activation domain. Therefore, we used a truncated form of *OsMADS1* as a bait. This truncated gene spanned amino acid residues 1–218 of *OsMADS1* containing the complete MADS box and the I and K domains but lacking the C-terminal part of the C domain (Figure 1A, (b)).

By two-hybrid screening of about 1000 000 yeast transformants, we obtained 13 positive clones. Sequencing revealed that these clones originated from two genes not previously known. We named the novel genes *OsMADS14* and *OsMADS15*. The cloned inserts contained different starting points as seen in Figure 2. Some of the clones lacked the MADS domain and most of the I domain (for example, Figure 2, No. 80), but all of the clones contained complete K and C domains. This suggests that the region essential for the *OsMADS1/OsMADS14* and *OsMADS1/OsMADS15* interactions lies downstream of the I domain.

We found an *OsMADS15* cDNA clone containing the entire open reading frame by 5′-RACE. The nucleotide sequence of this *OsMADS15* cDNA clone is shown in Figure 3A. The nucleotide sequence of *OsMADS14* has been published elsewhere (Moon *et al.*, 1999). The *OsMADS15* protein contains a MADS box domain of 56 amino acids at the N-terminus (Figure 3A, underlined 2–57). The K domain, a region considered to participate in protein-protein interaction, resides at the amino acids 93–158 (Figure 3A, underlined region). The *OsMADS15* protein shows high similarity to *OsMADS14* and *ZAP1* (Figure 3B), a maize homologue of *AP1* (Mena *et al.*, 1995). The amino acid sequence comparison of *OsMADS15* revealed 66% sequence identity and 67% sequence homology to *ZAP1* (Figure 3B). The sequences of *OsMADS1, OsMADS14, OsMADS15*, and *ZAP1* shared a high degree of homology in the MADS and K domains (Figure 3B). However, in the I and C regions, *OsMADS1* showed only little homology to *OsMADS14, -15*, and *ZAP1* (Figure 3B). *OsMADS14* and *-15* are highly homologous to each other throughout the protein region except for short deletions in several places of the C domain of *OsMADS14* in comparison to *OsMADS15* (see the underlined region in Figure 3B).

Determination of regions required for protein-protein interaction

The regions of *OsMADS1*4 and of *OsMADS15* responsible for the protein-protein interaction with *OsMADS1* were determined both in the yeast two-hybrid system and by *in vitro* pull-down assay. We focused on the K and C domains in this study, since all the *OsMADS14* and *-15* clones, selected in the two-hybrid system using the bait *OsMADS1*, contained both K and C domains (Figure 2).

Interestingly, two copies of the well known motif called a 'leucine zipper', which often exists in transcriptional activators calling for protein-protein interaction, are present in *OsMADS1, -14*, and *-15* as shown in Figure 4A (note the leucine or isoleucine in boxes). Forty-one amino acids are between the leucine zipper-like motifs (Figure 4A). We arbitrarily defined this region in *OsAMDS1, -14*, and *-15* as K′. It includes K and a following conserved segment of 16 amino acids with a second leucine zipper-like motif

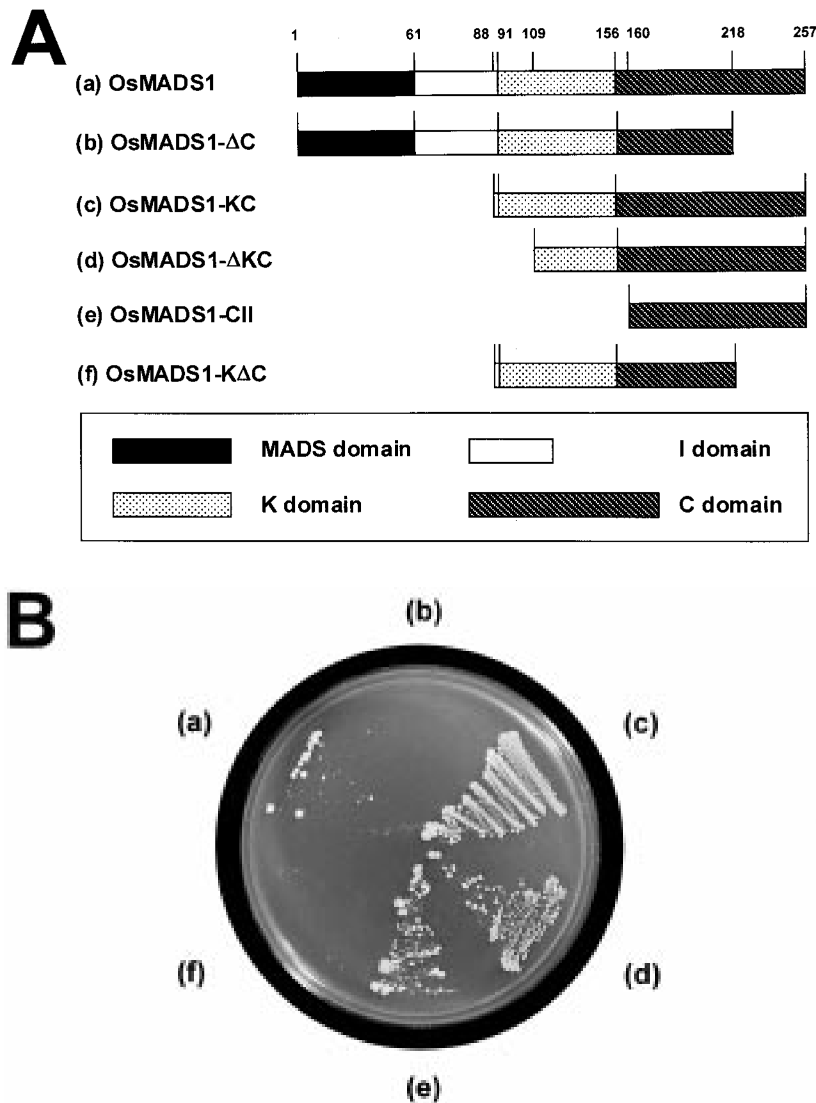

Figure 1. Determination of the transcriptional activation domain in *OsMADS1*. A. Schematic diagram of serial deletion mutants of *OsMADS1* were fused to the GAL4 DNA-binding domain. These plasmids contain different parts of *OsMADS1* (a, *OsMADS1*, 1–257; b, *OsMADS1*-ΔC, 1–218; c, *OsMADS1*-KC, 88–257; d, *OsMADS1*-ΔKC, 109–257; e, *OsMADS1*-CII, 160–257; f, *OsMADS1*-KΔC, 88–218). The numbers on the top of the panel indicate amino acid positions in the wild-type *OsMADS1* gene. Closed, open, gray, and hatched boxes depict the MADS, I, K, and C domains, respectively. B. The viability of yeast cells containing the plasmids shown in panel A on a histidine-deficient plate.

(Figure 3B and 4A). C domains starts at amino acid 157 in *OsMADS1* and 160 in *OsMADS14* and *-15* (Figure 3B).

In the yeast two-hybrid system, which is often used to investigate protein-protein interactions *in vivo*, the constructs of *OsMADS14* and *-15* containing K domains (K, K′, and KC) interacted with *OsMADS1* containing the KC domain as indicated by the growth of the yeast transformants on Leu-, Trp-, and His-deficient plates (Figure 4B, (a), (b), (c), (e), (f), and (g)). Note that the growth rate of the yeast cells transformed with *OsMADS14*-K and *OsMADS15*-K is lower than that of yeast cells transformed with *OsMADS14*-KC, *OsMADS14*-K′, *OsMADS15*-KC, and *OsMADS15*-K′ (compare (b) and (f) with (a), (c), (e), and (g) in Figure 4B). Derivatives of *OsMADS14* and *-15* lacking the K domain did not interact with *OsMADS1* (Figure 4B, (d) and (h)). This indicates that the presence of the K domains in both *OsMADS14* and *OsMADS15* is

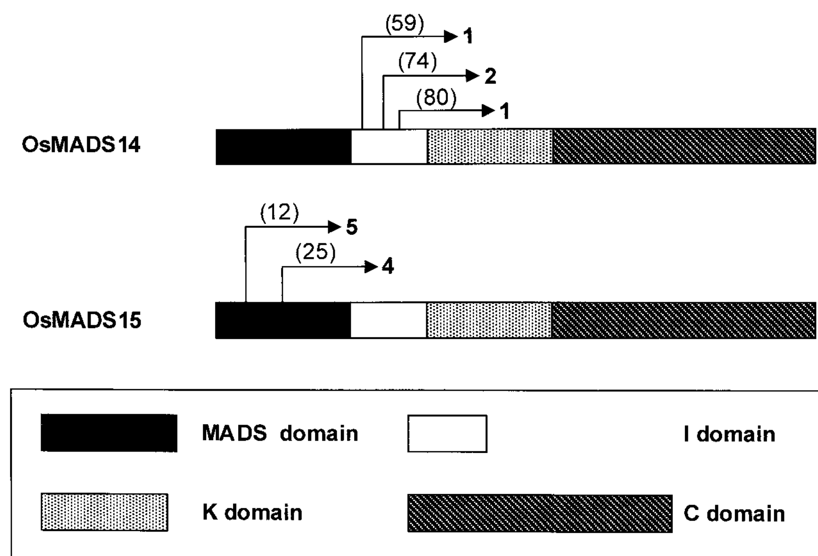

Figure 2. Rice cDNA clones selected in a yeast two-hybrid system using the *OsMADS1* gene as a bait. Schematic diagrams of the identified genes are shown. Closed, open, gray, and hatched boxes depict the MADS, I, K, and C domains, respectively. Two different genes (*OsMADS14* and *OsMADS15*) were repeatedly selected. Plasmid names and selected independent clones are indicated. The amino acid numbers of *OsMADS14* and *-15* are shown as numbers on arrows. Note that all clones contain K and C domains.

essential for the interaction with *OsMADS1*. We attempted to compare the relative strength of the protein-protein interactions among derivatives of *OsMADS14* and *-15* by measuring the β-galactosidase activities of the yeast transformants (Table 1). Admittedly, β-galactosidase activity may not quantitatively reflect the strength of the protein-protein interaction, but we still hoped to get a rough idea of the relative strength of the protein-protein interactions. Yeast cells containing pGBT9/*OsMADS1*-KΔC and pGAD424, a negative control pair, showed minimal β-galactosidase activity (0.67 units). A positive control pair, pGAD424 and pGBT9/AD, where the GAL4 DNA-binding domain is directly fused with the GAL4 transcriptional activation domain 11 (amino acids 768–881) (Ma and Ptashne, 1987), showed a β-galactosidase activity of about 55 units. The clones containing a K′ domain of *OsMADS14* (pGAD424/*OsMADS14*-KC) showed very strong interaction with *OsMADS1* judged by the strong β-galactosidase activity of about 49 units. Deletion of the C domain (pGAD424/*OsMADS14*-K′) reduced β-galactosidase activity about 5-fold (9.4 units). This may indicate that the C domain of *OsMADS14* augments the interaction between *OsMADS1* and *OsMADS14*. It is hard to speculate on the molecular basis for the enhancement of β-galactosidase activity by the C-domain with this limited experiment. Deletion of the second leucine zipper-like domain (PGAD424/*OsMADS14*-K) reduced the β-galactosidase activity further, even though the activity remained much higher than that of the clone containing the C domain alone (pGAD424/*OsMADS14*-C). This pattern was also observed for yeast cells containing derivatives of pGAD424/*OsMADS15*, although the β-galactosidase activities were generally lower than those of the *OsMADS14* clones. Our data therefore indicate that the K domains of *OsMADS14* and *OsMADS15* include the minimal sequences required for protein-protein interaction and that the second leucine zipper-like motif augments the interaction.

The interaction of *OsMADS14* and *-15* with *OsMADS1* was confirmed by *in vitro* pull-down assay. [^{35}S]-labeled *OsMADS14* and *-15* proteins were synthesized in an RRL *in vitro* translation system. GST and GST-fused *OsMADS1* proteins were produced in *E. coli* and partially purified using glutathione Sepharose 4B resin. The resin-bound GST and GST-fused *OsMADS1* proteins were mixed with [^{35}S]-labeled *OsMADS14* and *-15* proteins containing the K, K′, C, or KC regions. After thorough washing, the resin-bound proteins were analyzed by SDS-PAGE. The translation products of GAL4-AD, a negative control protein spanning the GAL4 activation domain, and of the *OsMADS14* and *-15* derivatives were clearly detected by autoradiography (Figure 4C, lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25). These proteins were

A

OsMADS15 cDNA

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  C TCC TCT TCT TCT TCT TCC ACT AGC TAG TTC GTC TTC CTC CTT CAG CTA GCT TGT AGC 58
AGC TAA GGT TAG GTC GGA TCG AGA TCG GGA TCG GCC GCC GGC GAG CGG CGA GCG GCG AGG 118

ATG GGG CGG GGG AAG GTG CAG CTG AAG CGG ATA GAG AAC AAG ATC AAC AGG CAG GTG ACG 178
 M   G   R   G   K   V   Q   L   K   R   I   E   N   K   I   N   R   Q   V   T  20

TTC TCC AAG AGG AGG AAT GGA TTG CTG AAG AAG GCG CAC GAG ATC TCC GTC CTC TGC GAC 238
 F   S   K   R   R   N   G   L   L   K   K   A   H   E   I   S   V   L   C   D  40

GCC GAG GTC GCC GCC ATC GTC TTC TCC CCC AAG GGC AAG CTC TAC GAG TAC GCC ACT GAC 298
 A   E   V   A   A   I   V   F   S   P   K   G   K   L   Y   E   Y   A   T   D  60

TCC AGG ATG GAC AAA ATC CTT GAA CGT TAT GAG CGC TAT TCA TAT GCT GAA AAG GCT CTT 358
 S   R   M   D   K   I   L   E   R   Y   E   R   Y   S   Y   A   E   K   A   L  80
                                                ***
ATT TCA GCT GAA TCC GAG AGT GAG GGA AAT TGG TGC CAT GAA TAC AGG AAA CTT AAG GCA 418
 I   S   A   E   S   E   S   E   G   N   W   C ▶H   E   Y   R   K   L   K   A 100

AAG ATT GAG ACC ATA CAA AAA TGT CAC AAA CAC CTC ATG GGA GAG GAT CAT GAA TCC CTG 478
 K   I   E   T   I   Q   K   C   H   K   H   L   M   G   E   D   H   E   S   L 120

AAT CTC AAA GAA CTC CAA CAG CTA GAG CAG CAG CTG GAG AGT TCA TTG AAG CAC ATA ATA 538
 N   L   K   E   L   Q   Q   L   E   Q   Q   L   E   S   S   L   K   H   I   I 140

TCA AGA AAG AGC CAC CTT ATG CTT GAG TCC ATT TCC GAG CTG CAG AAA AAG GAG AGG TCA 598
 S   R   K   S   H   L   M   L   E   S   I   S   E   L   Q   K   K   E   R   S 160
                                                    ***
CTG CAG GAG GAG AAC AAG GCT CTG CAG AAG GAA CTG GTG GAG AGG CAG AAG AAT GTG AGG 658
 L   Q   E   E   N   K   A   L   Q   K   E   L   V   E◀  R   Q   K   N   V   R 180

GGC CAG CAG CAA GTA GGG CAG TGG GAC CAA ACC CAG GTC CAG GCC CAG GCC CAA GCC CAA 718
 G   Q   Q   Q   V   G   Q   W   D   Q   T   Q   V   Q   A   Q   A   Q   A   Q 200

CCC CAA GCC CAG ACA AGC TCC TCC TCC TCC TCC ATG CTG AGG GAT CAG CAG GCA CTT CTT 778
 P   Q   A   Q   T   S   S   S   S   S   S   M   L   R   D   Q   Q   A   L   L 200

CCA CCA CAA AAT ATC TGC TAC CCG CCG GTG ATG ATG GGC GAG AGA AAT GAT GCG GCG GCG 838
 P   P   Q   N   I   C   Y   P   P   V   M   M   G   E   R   N   D   A   A   A 240

GCG GCG GCG GTG GCG GCG CAG GGC CAG GTG CAA CTC CGC ATC GGA GGT CTT CCG CCA TGG 898
 A   A   A   V   A   A   Q   G   Q   V   Q   L   R   I   G   G   L   p   p   w 260

ATG CTG AGC CAC CTC AAT GCT TAA GAT GAT CAT CGT CGT CGT CGT CGG CCA AAC AGC TGC  958
 M   L   S   H   L   N   A   *                                                  268
CGT ATG CAC CGT GAA TCA TGG GAG CAA CCT TGA ATG AAT TGA AGT CAT TGG TAT CGA TCC 1018
TAG CGA TAA TAT ATA TGA TTC TCC TAA AAT GAA ATT GAT CTC AAA AAA ACA AAC CTA GCG 1078
ATT AAG CTA TTC TTA TAT ATG TGT TTG CCT GCT GCC CCC TAC CCT ACA GGC TAC ATA TGA 1138
TTT GCA AGA AAT TAA TTA TGA GCA AGG ATC AGG ATG TGT CTT TGT GTA ATC ATC AGC ACG 1198
TAC CTA GTG CTT CTT ACT GAT ATA TAT GCA TGC AAT TGT GTG CAT ATA AAT ATA TTT GCA 1258
TGC CAA AAA AAA AAA AAA AAA AAA AAA                                             1285
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Figure 3. A. The nucleotide sequence and predicted amino acid sequence of the *OsMADS15* cDNA. The GenBank accession number is AF058698. MADS box and K box regions are underlined. The positions of nucleotides and amino acids are shown in the right margin. The asterisks (***) indicate the first and the last amino acid of the K′ domain of *OsMADS15*. B. Sequence homology comparison among *OsMADS1, -14, -15*, and *ZAP1*. Analysis of the amino acid homology was performed by the Macvector program. Identical amino acid residues and amino acids with conserved changes are depicted within darker and lighter boxes, respectively. MADS, K, K′, and C domains are indicated by arrows.

not pulled-down by the glutathione Sepharose 4B resin associated with GST (Figure 4C, lanes 2, 5, 8, 11, 14, 17, 20, 23, and 26). *OsMADS14* and *-15* proteins containing the K′ region (K′ and KC) were precipitated by glutathione Sepharose 4B resin only together with GST-*OsMADS1*-KΔC (Figure 4C, lanes 6, 12, 18, and 24). As already seen in the two-hybrid system, the C domain of *OsMADS14* and *-15* did not interact with *OsMADS1* in the pull-down assay either (Figure 4C, lanes 15 and 27). *OsMADS14* and *-15* proteins containing only the K domain showed some very weak interaction with *OsMADS1* in this pull-down experiment (Figure 4C, lanes 9 and 21). Overexposure of the X-ray film revealed faint bands of *OsMADS14* and *-15* (data not shown). This, too, suggests that the leucine zipper-like motif at the C-terminal ends of the

Figure 3. Continued.

Table 1. Quantitative analysis of protein-protein interactions among derivatives of OsMADS1, -14, and -15. β-galactosidase activity was determined by the ONPG assay.

plasmids	β-galactosidase activity[1]
pGBT9+pGAD424	0.67 ± 0.09
pGBT9/AD+pGAD424	55.13 ± 1.65
pGBT9/OSMADS1-KΔC+pGAD424/OsMADS14-KC	48.65 ± 2.96
pGBT9/OSMADS1-KΔC+pGAD424/OsMADS14-K	2.26 ± 0.25
pGBT9/OSMADS1-KΔC+pGAD424/OsMADS14-K′	9.40 ± 0.26
pGBT9/OSMADS1-KΔC+pGAD424/OsMADS14-C	0.90 ± 0.07
pGBT9/OSMADS1-KΔC+PGAD424/OsMADS15-KC	8.96 ± 1.28
pGBT9/OSMADS1-KΔC+PGAD424/OsMADS15-K	1.27 ± 0.16
pGBT9/OSMADS1-KΔC+pGAD424/OsMADS15-K′	3.39 ± 0.30
pGBT9/OSMADS1-KΔC+pGAD424/OsMADs15-C	0.77 ± 0.12

[1] β-galactosidase activity: 1 unit = $1000 \times A_{420}/(A_{600} \times$ reaction time (min) $\times$ volume of culture (ml). Three independent assays were performed for each yeast transformation. Mean $\pm$ standard deviation.

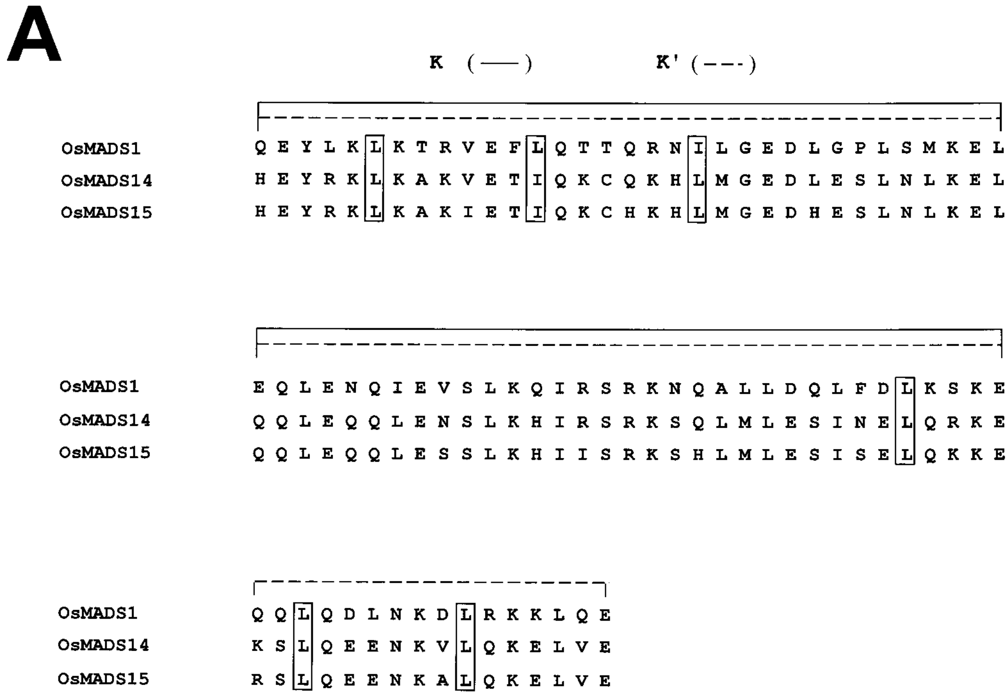


Figure 4. Determination of the domains in *OsMADS14* and *-15* responsible for interaction with *OsMADS1*. A. The amino acid sequences of the K′ regions of *OsMADS1, -14*, and *-15*. Leucine or isoleucine residues, which are repeated at intervals of seven amino acid residues, are boxed-in. B. The viability test of yeast cells transformed by two-hybrid vectors on a histidine-deficient plate. *OsMADS14*-KC, -K, -K′, and -C encode amino acids 93–246, 93–158, 93–174, and 159–246 of *OsMADS14*, respectively. *OsMADS15*-KC, -K, -K′ and -C encode amino acids 93–268, 93–158, 93–174, and 159–268 of *OsMADS15*, respectively. C. *In vitro* binding of *OsMADS14* and *-15* derivatives to *OsMADS1*. *In vitro* translation products of the GAL4 activation domain, *OsMADS15*-KC, -K, -K′, -C, and *OsMADS14*-KC, -K, -K′, -C are shown in lanes 1, 4, 7, 10, 13, 16, 19, 22, and 25, respectively. The same set of polypeptides precipitated with glutathione Sepharose 4B resins bound to GST protein is shown in lanes 2, 5, 8, 11, 14, 17, 20, 23, and 26, respectively. The same set of polypeptides precipitated with glutathione Sepharose 4B resins bound to the GST-fused *OsMADS1*-KΔC protein is shown in lanes 3, 6, 9, 12, 15, 18, 21, 24, and 27, respectively.

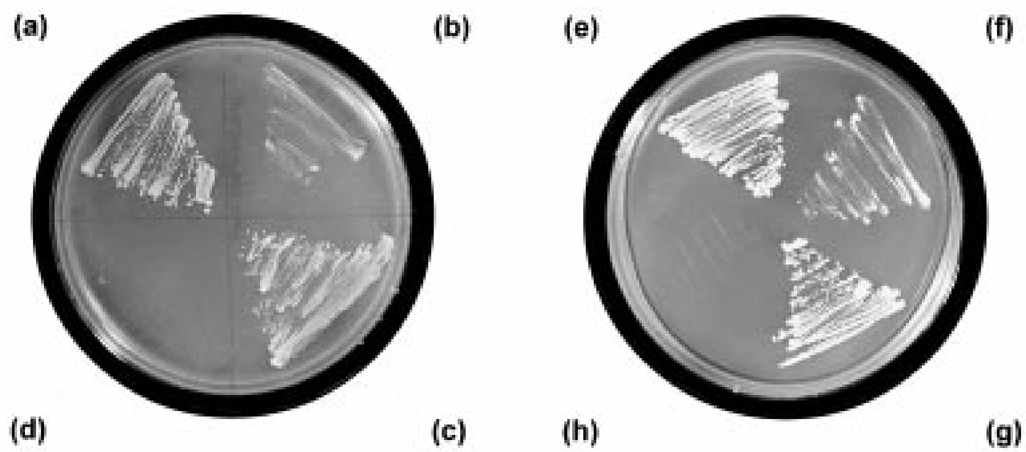
K′ domain of *OsMADS14* and *-15* strongly augment the protein-protein interaction.

Transcriptional activator functions of OsMADS1, -14*, and* -15.

Recently, the C region of *A. thaliana AP1* was shown to function as a transcriptional activation domain (Cho *et al.*, 1999). The possible transcriptional activator function of the C domain or KC domain in *OsMADS1, -14*, and *-15* was investigated using yeast and mammalian systems evaluating transcriptional activator function. Like in the *A. thaliana AP1*, the C domain of *OsMADS1* showed transcriptional activator function as indicated by the growth of yeast cells on a histidine-deficient plate (Figure 1B, (a), (c), (d) and (e)). The C domains of *OsMADS14* and *-15* did not show transcriptional activator function in yeast cells (data not show). Transcriptional activator function of *OsMADS1, -14* and *-15* was also investigated in a mammalian system (Figure 5A and 5B). The KC domain of *OsMADS1* showed transcriptional activator function in mammalian cells as indicated by CAT activity (Figure 5A and 5B, compare (c) with a negative control, (a)). On the other hand, the KC domains of *OsMADS14* and *-15* did not show transcriptional activator function (Figure 5A and 5B, compare (d) and (e) with (a)).

Discussion

OsMADS1 is a MADS box gene that is expressed uniformly in young flower primordia and then becomes localized in the palea, lemma, and ovary at a later stage of flower development (Chung *et al.*, 1994). Ectopic expression of *OsMADS1* with the CaMV 35S promoter in transgenic tobacco plants dramatically altered the development of the plant, creating short, bushy, early-flowering plants with reduced apical dominance.



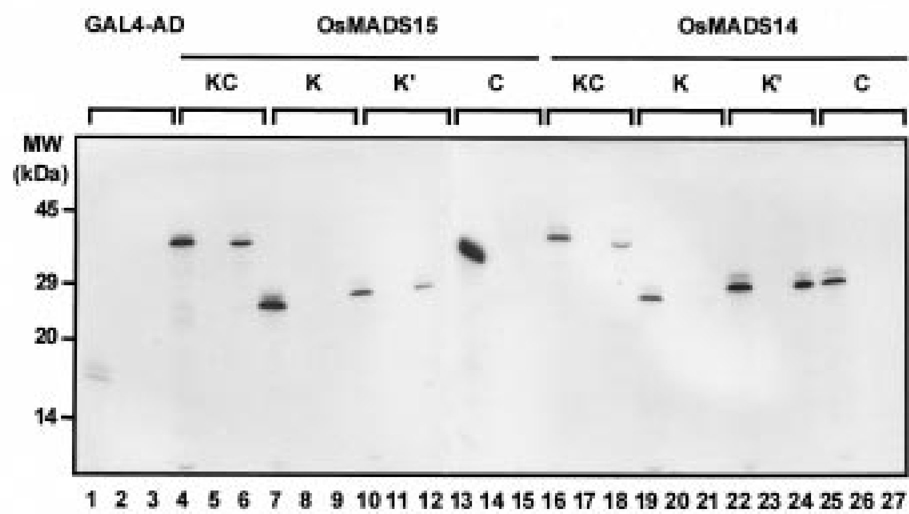


Figure 4. Continued.

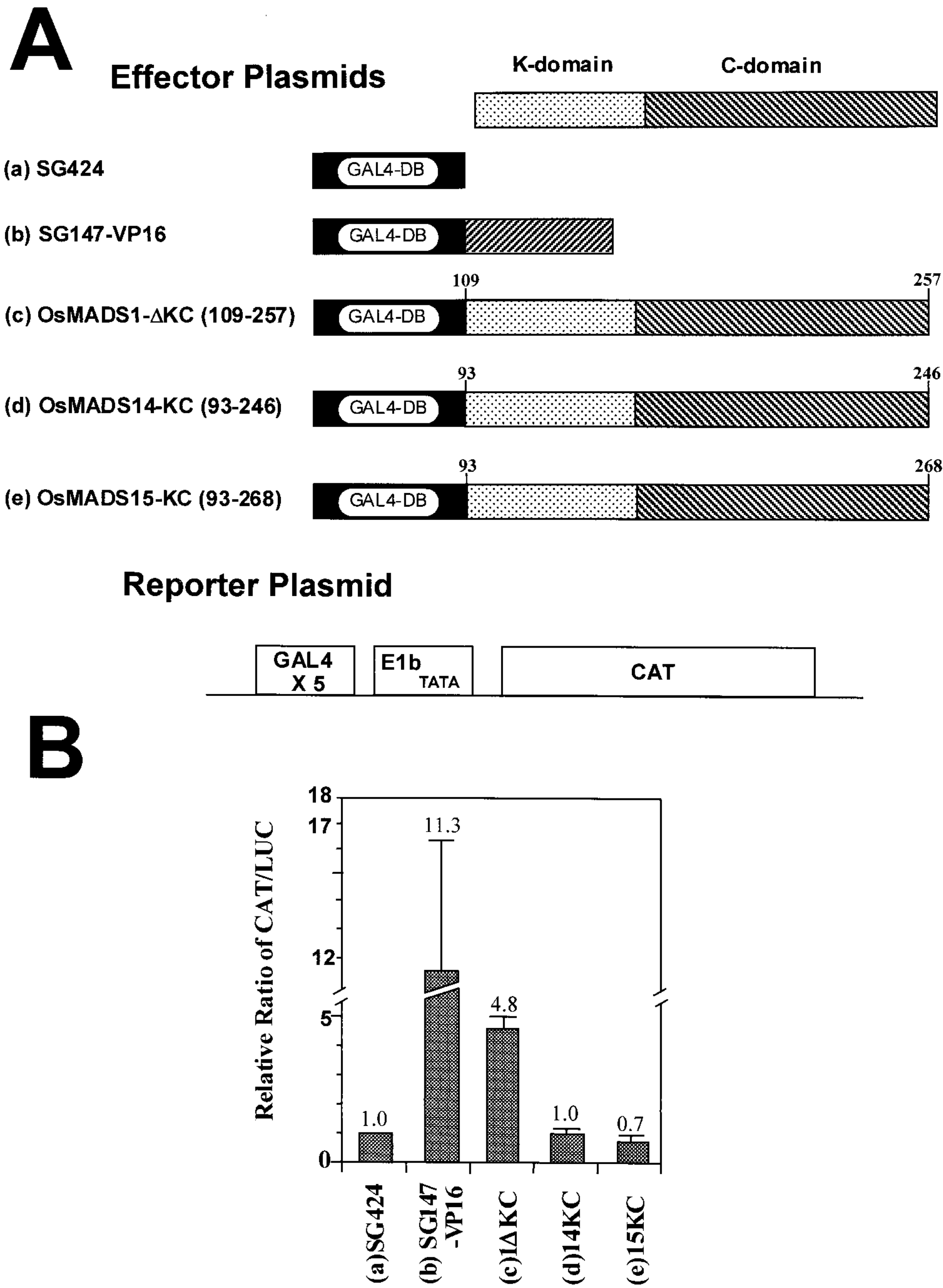


Figure 5. Transcriptional activator function of *OsMADS1, -14* and *-15* in mammalian cells. A. Schematic diagrams of portions of *OsMADS1 -14*, and *-15* that were used to investigate the transcriptional activation function of the regions in mammalian cells. The numbers on the top of the panel are amino acid numbers of the *OsMADS* genes. Gray and hatched boxes depict K and C domains, respectively. The reporter plasmid contains 5 *GAL4*-binding sites (GAL4 ×5), the adenovirus E1b promoter (E1b TATA), and the CAT (CAT) gene. Transcriptional activator function of the KC domains of *OsMADS1, -14*, and *-15* in mammalian cells was investigated by transient expression of test molecules in COS-7 cells. B. Relative transcriptional activation function of *OsMADS1*ΔKC, *14*KC and *15*KC in mammalian cells. Bars in the histogram show average CAT activities in mammalian cells transfected with the plasmids shown in the panel A. CAT activities in the cells transfected with the negative control vector pSG424 were arbitrarily set at 1. The columns represent mean values, and error bars indicate standard deviations. The data were normalized for transfection efficiency based on luciferase activity derived from the co-transfected plasmid pGL2. Three independent assays were performed for each transfection.

The results thus suggest that *OsMADS1* plays a key role in flower induction (Chung *et al.*, 1994). Here we report that a central portion of *OsMADS1*, named K′, is involved in interactions of *OsMADS1* with two novel MADS domain proteins named *OsMADS14* and *-15*. Furthermore, the C-terminal region of *OsMADS1* can function as a transcriptional activation domain in yeast and mammalian systems.

Several lines of evidence suggest that the transcriptional activation domains found in the KC region of the *OsMADS1* sequence function as such in plant cells, but this activity has yet to be confirmed for the *OsMADS1* protein in a plant. Many transcriptional activators are composed of a DNA-binding domain and a transcriptional activation domain (Brent and Ptashne, 1985; Hope and Struhl, 1986). The C region of a plant MADS box gene, *AP1*, has been shown to function as a transcriptional activator domain (Cho *et al.*, 1999). Therefore, it would not be surprising to find that *OsMADS1* could also function as a transcriptional activator, even though the similarity between the C domains of *OsMADS1* and *AP1* is not that high (Figure 3B). Furthermore, transcriptional activator sequences are well conserved between mammalian, plant, and yeast cells (Hope and Struhl, 1986; Ma and Ptashne, 1987; Courey *et al.*, 1989; Mermod *et al.*, 1989; Aeschbacher *et al.*, 1991; Schindler *et al.*, 1992), so much so that a transcriptional activator of one group can function in a very different group of organisms (Fischer *et al.*, 1988; Kakidani and Ptashne, 1988; Lech *et al.*, 1988; Ma *et al.*, 1988; Struhl, 1988; Webster *et al.*, 1988).

Two MADS box genes (*OsMADS14* and *-15*) were identified by their interaction with *OsMADS1* in the yeast two-hybrid system. We think these two proteins are major *OsMADS1*-binding proteins expressed at the early stage of rice flower development, since only these genes were repeatedly found during the two-hybrid screening process using a cDNA library generated from mRNAs of young rice flowers (4 for *OsMADS14*, and 9 for *OsMADS15*).

The K domain has been shown to play a key role in the interactions of other plant MADS domain proteins (Davies *et al.*, 1996; Fan *et al.*, 1997; West *et al.*, 1998). Interestingly, the K domain and the following sequences in *OsMADS14* and *-15*, named K′, seemed to be critical for the interaction *OsMADS1* with *OsMADS14* and *-15* in the two-hybrid system (Figure 4B) as well as the *in vitro* pull-down assay (Figure 4C). The C-terminal end of the K′ region containing the second leucine zipper-like motif, which is often found in domains responsible for protein-protein interaction of transcriptional activators (Landschulz *et al.*, 1988; Kouzarides and Ziff, 1988), visibly augmented the protein-protein interaction especially in the *in vitro* binding assay (Figure 4C). The configuration of the three proteins *OsMADS1, -14*, and *-15* in terms of the DNA-binding region (MADS domain), the protein-interaction region (K domain), and the leucine zipper-like domain is similar to that of the transcription activator Myc which contains a DNA-binding region (basic region), a protein-interaction domain (helix-loop-helix region), and a leucine zipper domain (Lucher and Larsson, 1999). Recently, we confirmed the importance of the leucine residues for protein-protein interaction in a MADS domain protein, *OsMADS6*, using site-directed mutagenesis (Moon *et al.*, 1999).

What roles could there be for protein-protein interactions among MADS domain proteins? Depending on the protein-interaction domains, MADS domain proteins may form different protein complexes. The different complexes are likely to have different DNA-binding specificities to different variants of target DNAs called CArG box motifs, even though the complexes may retain some binding capacity to the consensus (CC[A/T]$_6$GG) sequence. The variable C domains seem to have a different function, such as transcriptional activation. For instance, the KC region of *OsMADS1* but not those of *OsMADS14* and *-15* show transcriptional activator function. In different combinations the MADS domain proteins may be able to regulate the expression of a variety of target genes in spatially and temporally separated compartments.

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References

Aeschbacher, RA., Schrott, M., Potrykus, I. and Saul, M.W. 1991. Isolation and molecular characterization of PosF21, an *Arabidopsis thaliana* gene which shows characteristics of a b-Zip class transcription factor. Plant J. 1: 303–316.

Angenent, G.C., Busscher, M., Franken, J., Mol, J.N.M. and van Tunen, A.J. 1992. Differential expression of two MADS box genes in wild-type and mutant petunia flowers. Plant Cell 4: 983–993.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N. and Coen, E. 1993. Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of *Antirrhinum*. Cell 72: 85–5.

Brent, R. and Ptashne, M. 1985. A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. Nature 314: 198.

Cho, S., Jang, S., Chae, S., Chung, K.M., Moon, Y.H., An, G. and Jang, S.K. 1999. Analysis of the C-terminal region of *Arabidopsis thaliana APETALA1* as a transcription activation domain. Plant Mol. Biol. 40: 419–429.

Chung, Y.Y., Kim, S.R., Finkel, D., Yanofsky, M.F. and An, G. 1994. Early flowering and reduced apical dominance result from ectopic expression of a rice MADS box gene. Plant Mol. Biol. 26: 657–665.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G. and Carpenter, R. 1990. *floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. Cell 63: 1311–1322.

Courey, A.J., Holtzman, D.A., Jackson, S.P. and Tjian, R. 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell 1: 827–836.

Davies, B., Egea-Cortines, M., de Andrade Silva, E., Saedler, H. and Sommer, H. 1996. Multiple interactions amongst floral homeotic MADS box proteins. EMBO J. 15: 4330–4343.

Fan, H.Y., Hu, Y., Tudor, M. and Ma, H. 1997. Specific interactions between the K domains of *AG* and *AGLs*, members of the MADS domain family of DNA binding proteins. Plant J. 12: 999–1010.

Feilotter, H.E., Hannon, G.J., Ruddell, C.J. and Beach, D. 1994. Construction of an improved host strain for two-hybrid screening. Nucl. Acids Res. 22: 1502–1053.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interactions. Nature 20: 245–246.

Fischer, J.A., Giniger, E., Maniatis, T. and Ptashne, M. 1988. GAL4 activates transcription in *Drosophila*. Nature 332: 853–856.

Flanagan, C.A. and Ma, H. 1994. Spatially and temporally regulated expression of the MADS-box gene *AGL2* in wild-type and mutant *Arabidopsis* flowers. Plant Mol. Biol. 26: 581–595.

Gietz, D., St Jean, A., Woods, RA. and Schiestl, R.H. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. 25: 1425.

Greco, R., Stagi, L., Colombo, L., Angenent, G.C., Sari-Gorla, M. and Pe, M.E. 1997. MADS box genes expressed in developing inflorescences of rice and sorghum. Mol. Gen. Genet. 20: 615–623.

Hayes, T.E., Sengupta, P. and Cochran, B.H. 1988. The human c-fos serum response factor and the yeast factors GRM/PRTF have related DNA-binding specificities. Genes Dev. 2: 1713–1722.

Hirai, S., Bourachot, B. and Yaniv, M. 1990. Both Jun and Fos contribute to transcription activation by the heterodimer. Oncogene 5: 39–76.

Hope, I.A. and Struhl, K. 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell 12: 885–894.

Huijser, PW., Klein, J., Lonnig, W-E., Meijer, H., Saedler, H. and Sommer, H. 1992. Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in *Antirrhinum majus*. EMBO J. 11: 1239–1249.

Jack, T., Brochman, L.L. and Meyerowitz, F.M. 1992. The homeotic gene APETALA3 of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 68: 683–697.

Kakidani, H. and Ptashne, M. 1988. GALA activates gene expression in mammalian cells. Cell 29: 161–167.

Kang, H.G. and An, G. 1997. Isolation and characterization of a rice MADS box gene belonging to the *AGL2* gene family. Mol. Cells 28: 45–51.

Kang, H.G., Noh, Y.S., Chung, Y.Y., Costa, M.A., An, K. and An, G. 1995. Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. Plant Mol. Biol. 29: 1–10.

Kang, H.G., Jang, S., Chung, J.E., Cho, Y.G. and An, G. 1997. Characterization of two rice MADS box genes that control flowering time. Mol. Cells 31: 559–566.

Kempin, S.A., Mandel, M.A. and Yanofsky, M.F 1993. Conversion of perianth into reproductive organs by ectopic expression of the tobacco floral homeotic gene NAG1. Pant Physiol. 103: 1041–1046.

Kouzarides, T. and Ziff, E. 1988. The role of the leucine zipper in the fos-jun interaction. Nature 15: 646–651.

Landschulz, W.H., Johnson, PF and McKnight, S.L. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 24: 1759–1764.

Lech, K., Anderson, K. and Brent, R. 1988. DNA-bound Fos proteins activate transcription in yeast. Cell 29: 179–184.

Lu, Z.X., Wu, M., Loh, CS., Yeong, C.Y. and Gob, C.J. 1993. Nucleotide sequence of a flower-specific MADS box cDNA clone from orchid. Plant Mol. Biol. 23: 901–904.

Luscher, B. and Larsson, L.G. 1999. The basic region/helix-loop-helix/leucine zipper domain of Myc pronto-oncoproteins: function and regulation. Oncogene 13: 2955–2966.

Ma, J. and Ptashne, M. 1987. A new class of yeast transcriptional activators. Cell 9: 113–119.

Ma, J., Przibilla, E., Hu, J., Bogorad, L. and Ptashne, M. 1988. Yeast activators stimulate plant gene expression. Nature 18: 631–633.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. 1991. *AGL1–AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. 5: 484–495.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. 1992. Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. Nature 360: 273–277.

McGonigle, B., Bouhidel, K. and Irish, V.F. 1996. Nuclear localization of the *Arabidopsis APETALA3* and *PISTILLATA* homeotic gene products depends on their simultaneous expression. Genes Dev. 15: 1812–1821.

Mena, M., Mandel, M.A., Lemer, D.R., Yanofsky, M.V. and Schmidt, R.J. 1995. A characterization of the MADS-box gene family in maize. Plant J. 8: 845–854.

Mermod, N., O'Neill, E.A., Kelly, T.J. and Tjian, R. 1989. The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. Cell 25: 741–753.

Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Montag, K., Salamini, F and Thompson, R.D. 1995. ZEMa, a member of a novel group of MADS box genes, is alternatively spliced in maize endosperm. Nucl. Acids Res. 25: 2168–2177.

Moon, Y.H., Kang, HG., Jung, J.Y., Jeon, J.S., Sung, S.K. and An, G. 1999. Determination of the motif responsible for interaction between the rice *APETALA1/AGAMOUS-LIKE9* family proteins using a yeast two-hybrid system. Plant Physiol. 120: 1193–1204.

Neumann, J.R., Morency, C.A. and Russian, K.O. 1987. A novel rapid assay for chloramphenicol acetyl transferase gene expression. BioTechniques 5: 444-447.

Norman, C., Runswick, M., Pollock, R. and Treisman, R. 1988. Isolation and properties of cDNA clones encoding SRF, a tran-

scription factor that binds to the c-fos serum response element. Cell 23: 989–1003.

Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, A. and Lifschitz, F. 1991. The MADS box gene family in tomato: temporal expression during floral development, converted secondary structures and homology with homeotic genes from *Antirrhinum* and *Arabidopsis*. Plant J. 1: 255–266.

Ponticelli, A.S., Pardee, T.S. and Struhl, K. 1995. The glutamine-rich activation domains of human Sp1 do not stimulate transcription in *Saccharomyces cerevisiae*. Mol. Cell Biol. 15: 983–988.

Purugganan, M.D., Rounsley, S.D., Schmidt, R.J. and Yanofsky, M.F. 1995. Molecular evolution of flower development: diversification of the plant MADS-box regulatory gene family. Genetics 140: 345–356.

Reese, J.C., Apone, L., Walker, S.S., Griffin, L.A. and Green, M.R. 1994. Yeast TAVIIs in a multisubunit complex required for activated transcription. Nature 371: 523–527.

Riechmann, J.L, Krizek, B.A. and Meyerowitz, E.M. 1996. Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins *APETALA1, APETALA3, PISTILLATA*, and *AGAMOUS* Proc Natl. Acad. Sci. USA 93: 4063-4070.

Riechmann, J.L., Wang, M. and Meyerowitz, E.M. 1996. DNA-binding properties of *Arabidopsis* MADS domain homeotic proteins *APETALA1, APETALA3, PISTILLATA*, and *AGAMOUS* Nucl. Acids Res. 24: 3134–3141.

Sambrook, J., Fritsch, E.F. and Manatees, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463–5467.

Savidge, B., Rounsley, S.D. and Yanofsky, M.V. 1995. Temporal relationship between the transcription of two *Arabidopsis* MADS box genes and the floral organ identity genes. Plant Cell 7: 721–733.

Schindler, U., Terzaghi, W., Beckmann, H., Kadesch, T. and Cashmore, A.R. 1992. DNA binding site preferences and transcriptional activation properties of *Arabidopsis* transcription factor GBF1. EMBO J. 11: 1275–1289.

Schmidt, R.J., Velt, B., Mandel, M.A., Mena, M., Hake, S. and Yanofsky, M.F. 1993. Identification and molecular charaterization of *ZAG1*, the maize homologue of *Arabidopsis* floral homeotic gene *AGAMOUS*. Plant Cell 5: 729–737.

Sommer H, Beltran J-P, Huijser P, Pape H, Lonnig, W-E., Saedler, H. and Schwarz-Sommer, Z. 1990. *Deficiens*, a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus*: the protein shows homology to transcription factors. EMBO J. 9: 605–613.

Struhl, K. 1988. The JUN oncoprotein, a vertebrate transcription factor, activates transcription in yeast. Nature 14: 649–650.

Tatsuka, M., Orita, S., Yagi, T., Kakunaga, T. 1988. An improved method of electroporation for introducing biologically active foreign genes into cultured mammalian cells. Exp. Cell Res. 178: 154–162.

Trobner, W., Ramirez, L., Motte, R, Hue, I., Huijser, R, Lonnig, W-E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. 1990. *GLOBOSA*, a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. EMBO J. 11: 4693–4704.

Webster, N., Jin, J.R., Green, S., Hollis, M. and Chambon, P. 1988. The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. Cell 52: 169–178.

Weigel, D. and Meyerowitz, E.M. 1994. The ABCs of floral homeotic genes. Cell 29: 203–209.

West, A.G., Causier, B.E., Davies, B. and Sharrocks, A.D. 1998. DNA binding and dimerisation determinants of *Antirrhinum majus* MADS-box transcription factors. Nucl. Acids Res. 1: 5277–5287.