Controlling Flower Development by MADS Box Genes

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Introduction

Life cycle of plants consists of two distinctive growth phases. Seed develops into leaf and root which are the vegetative parts of plants. Once plants grow to a certain stage, environmental signals induce a sudden change of growing apex to inflorescense meristem. This is the beginning step of a reproductive growth phase. Inflorescense meristem further develops to a floral primordium which differentiates into four distinctive organs: sepal, petal, stamen, and carpel. After fertilization, ovules in carpel growth to mature seeds.

It has been recognized for a long time that flowering time is dependent on day length in many plant species. A chemical signal which is believed to be generated in leaves during the night period may play an important role in controlling induction of flowering. However, the nature of the signal molecule and the procedure of flower induction are remained to be unknown.

Recently, a rapid progress has been made toward elucidating the underlymechanisms controlling flower development in distantly related dicot plant species (5, 8). These studies led to the isolation of a family of genes which encode regulatory proteins. These include *AGAMOUS (AG) (30), APETALA1 (AP1) (15), and APETALA3 (AP3) (11) in Arabidopsis thaliana, and DEFICIENS A (DEF A) (25), GLOBOSA (GLO) (27), SQUAMOSA (SQUA) (10),* and *PLENA (PLE)* (4) in *Antirrhinum majus*. Mutations in an *AG* or *PLE* gene resulted in homeotic alterations of stamen and carpel. Genetic studies have shown that *DEF A, GLO* and *AP3* genes are essential for petal and stamen development. *AP1* and *SQUA* genes which are expressed in young flower primordia are necessary for transition of an inflorescence meristem into a floral meristem. Sequence analysis of these genes revealed that the gene products contain a conserved MADS box region (4, 10, 11, 15, 25, 27, 30) which is probably a DNA-binding domain (24). Using these clones as probes, MADS box genes have also been isolated from other species including tomato (17), tobacco (12), petunia (2), *Brassica napus* (14), and maize (23).

Transgenic approaches were undertaken to study the functional roles of the MADS box genes. Genetic complementation of the ag-2 mutant by the *AG* gene demonstrated that the gene product is involved in stamen and carpel development (30). Ectopic expression of the *AG* genes from *A. thaliana, B. napus*, petunia, tobacco, and tomato resulted in homeotic conversion of sepal to carpel and petal to stamen, mirroring the *ap2* mutant phenotype (12, 14, 16, 19, 28). These results support the hypothesis that *AG* and *AP2* act in an antagonistic fashion. Antisense approaches were also used to reveal the functional role of the tomato MADS box genes (18, 19). Transgenic plants that express tomato AG antisense RNA displayed the ag mutant phenotypes. Antisense expression of the tomato *TM5* MADS box gene resulted in morphological changes in

the three inner whorls of transgenic plants. In this current study we report the isolation of a rice MADS box homologue and the effects of its expression in tobacco plants.

Isolation of a rice cDNA clone encoding MADS box protein

We have isolated a cDNA clone, OsMADS1, by screening a λ ZapII cDNA library prepared from immature rice flower mRNA using mixed probes of different MADS box cDNA clones isolated from *Arabidopsis* (13, 30), *Brassica* (14), tobacco (12), and tomato (17). DNA sequence analysis showed that the rice clone encodes a putative protein of 257 amino acid residues (Fig. 1). The deduced amino acid sequence contains the conserved MADS box domain between the amino acids 2 and 57 (Fig. 2). A second domain, called the K box, present in the MADS box proteins is located between the residues 90 and 143. These observations suggest that OsMADS1 is a member of the MADS box gene family. Among characterized MADS box proteins, the OsMADS1 amino acid sequence is most homologous to *API* (44.4% identity) and *SQUA* (42.6% identity). In addition, OsMADS1 shows extensive similarity to the functionally anonymous *Arabidopsis* MADS box genes, AGL2 (56.2% identity) and AGL4 (55.4% identity).

Southern blot analysis

To determine the number of MADS box genes present in rice, DNA gel blot analysis was performed. The result showed that more than ten restriction fragments hybridized with the entire cDNA probe whereas a single fragment was detected by a probe lacking the conserved MADS box region (data not shown). This result indicates that the rice genome contains a high number of genes encoding MADS box proteins, similar to other plant species (2, 13, 17, 23).

Expression pattern of OsMADS1

Northern blot analyses were conducted to study the expression pattern of the OsMADS1 gene (Fig. 3). RNA samples were hybridized with the probe lacking the conserved MADS box region to avoid cross hybridization and obtain specific expression pattern of the gene. The OsMADS1 transcripts were present in palea, lemma, and carpel, but not in anther or vegetative organs (Fig. 3A). The gene was active during young inflorescence stage and the expression continued into the early and late vacuolated pollen stage (Fig. 3B). *In situ* experiments revealed that the OsMADS1 transcript was uniformly present in young flower primordia during early flower development and later became localized in certain floral organs (Fig. 4). In young inflorescence, strong hybridization signals were detected in flower primordia but not in other tissues. In vacuolated pollen-stage flower, OsMADS1 RNA was detected in palea, lemma, and ovary. However, hybridization signal was not uniform in these tissues. In particular, the tissues near the palea/lemma junction and the palea tissues covered by lemma exhibited little or no expression of the gene. No significant signal was observed from anther, filament, or sterile lemma. These results indicate that the OsMADS1 gene is preferentially expressed in certain floral tissues as has been observed with most MADS box genes.

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   1 AAAACTAGCTTGCAAAGGGGATAGAGTAGTAGAGAGAGAGAGAGAGGAGAGGAGGAGGAA

  61 GAAGATGGGGAGGGGGAAGGTGGAGCTGAAGCGGATCGAGAACAAGATCAGCCGGCAGGT
       MetGlyArgGlyLysValGluLeuLysArgIleGluAsnLysIleSerArgGlnVa  19
                                                          MADS
 121 GACGTTCGCCAAGCGCAGGAACGGCCTGCTCAAGAAGGCCTACGAGCTCTCCCTCCTCTG      -box
      lThrPheAlaLysArgArgAsnGlyLeuLeuLysLysAlaTyrGluLeuSerLeuLeuCy  39

 181 CGACGCCGAGGTCGCCCTCATCATCTTCTCCGGCCGCGGCCGCCTCTTCGAGTTCTCCAG
      sAspAlaGluValAlaLeuIleIlePheSerGlyArgGlyArgLeuPheGluPheSerSe  59

 241 CTCATCATGCATGTACAAAACCTTGGAGAGGTACCGCAGCTGCAACTACAACTCACAGGA
      rSerSerCysMetTyrLysThrLeuGluArgTyrArgSerCysAsnTyrAsnSerGlnAs  79

 301 TGCAGCAGCTCCAGAAAACGAAATTAATTACCAAGAATACCTGAAGCTGAAAACAAGAGT
      pAlaAlaAlaProGluAsnGluIleAsnTyrGlnGluTyrLeuLysLeuLysThrArgVa  99

 361 TGAATTTCTTCAAACCACACAGAGAAATATTCTTGGTGAGGATTTGGGCCCACTAAGCAT
      lGluPheLeuGlnThrThrGlnArgAsnIleLeuGlyGluAspLeuGlyProLeuSerMe 119
                                                          K-box
 421 GAAGGAGCTGGAGCAGCTTGAGAACCAGATAGAAGTATCCCTCAAACAAATCAGGTCAAG
      tLysGluLeuGluGlnLeuGluAsnGlnIleGluValSerLeuLysGlnIleArgSerAr 139

 481 AAAGAACCAAGCACTGCTTGATCAGCTGTTTGATCTGAAGAGCAAGGAGCAACAGCTGCA
      gLysAsnGlnAlaLeuLeuAspGlnLeuPheAspLeuLysSerLysGluGlnGlnLeuGl 159

 541 AGATCTCAACAAAGACTTGAGGAAAAAGTTACAGGAAACCAGTGCAGAGAATGTGCTCCA
      nAspLeuAsnLysAspLeuArgLysLysLeuGlnGluThrSerAlaGluAsnValLeuHi 179

 601 TATGTCCTGGCAAGATGGTGGTGGGCACAGCGGTTCTAGCACTGTTCTTGCTGATCAGCC
      sMetSerTrpGlnAspGlyGlyGlyHisSerGlySerSerThrValLeuAlaAspGlnPr 199

 661 TCATCACCATCAGGGTCTTCTCCACCCTCACCCAGATCAGGGTGACCATTCCCTGCAGAT
      oHisHisHisGlnGlyLeuLeuHisProHisProAspGlnGlyAspHisSerLeuGlnIl 219

 721 TGGGTATCATCACCCTCATGCTCACCATCACCAGGCCTACATGGACCATCTGAGCAATGA
      eGlyTyrHisHisProHisAlaHisHisHisGlnAlaTyrMetAspHisLeuSerAsnGl 239

 781 AGCAGCAGACATGGTTGCTCATCACCCCAATGAACACATCCCATCCGGCTGGATATGATG
      uAlaAlaAspMetValAlaHisHisProAsnGluHisIleProSerGlyTrpIle***   257

 841 TGTGTGTTCAGTTCAGGCTTCAGGCTTCAGAGAAGCCAATGCAAACAGTGTCCTGTAATC

 901 CAGTAATTACAGGGCATATGTAATGTAATGTAATGTAATCCCTGATCTATATTTTGCTAA

 961 GTACGTGCGTGCTCTCTTACGACCTTCTCCCCCAAACAGTTAATCAGGGGAATAATAATT

1021 TCGTTTGATGCACGTACTGTATGTCTGTATCTGTCACTGTATCGTAGGACCGTCCATGTA

1081 TAACAATTTCCGTTTTGGATGTGGTAACAATTAATTGGCACTTAAATTTATATTTGTGAT

1141 G(A)n
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Fig. 1. Nucleotide and deduced amino acid sequences of OsMADS1 cDNA. Both strands of the OsMADS1 cDNA were sequenced by the dideoxynucleotide chain termination method using double-stranded DNA as a template (22). MADS box and K box regions are underlined. The positions of nucleotides and amino acids are shown on the left and right, respectively.

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GRGKVELKRIENKISRQVTFAKRRNGLLKKAYELSLLCDAEVALIIFSGRGRLFEF   OsMADS1

***R*Q********N*****S***A******H*I*V********VV**HK*K***Y   AP1
*****Q********N*****S***G******H***V*********V**NK*K***Y   SQUA

****I*I*****TTN*****C**************V*********V**S****Y*Y   AG
****I*I*****ITN*****C**************V*********VV**S****Y*Y   PLE

*G*R*Q******QTN****YS******F***H**TV****R*SI*M**SSNK*H*Y   AP3
A***IQI*****QTN****YS******F***H***V****K*SI*MI*STQK*H*Y   DEF A
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Fig. 2. Comparison of MADS box regions. Alignment of OsMADS1 (residues 2-57) with other MADS box proteins; AP1, SQUA, AG, PLE, AP3, DEF A. The asterisks indicate identical amino acid to OsMADS1.

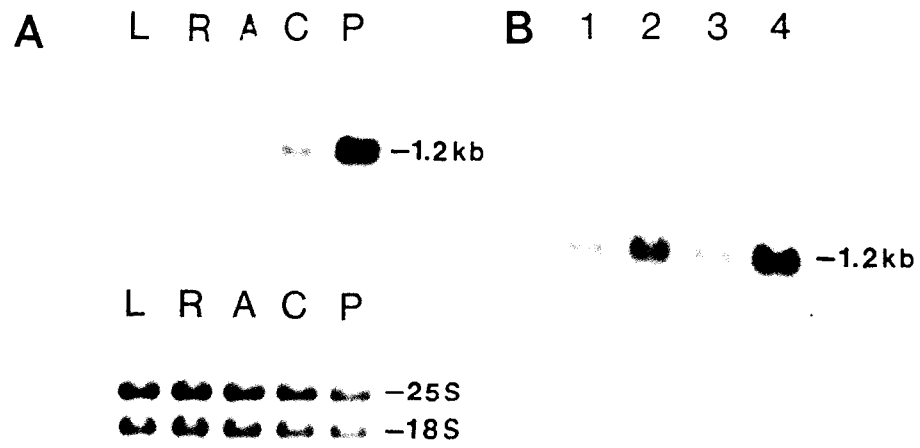

Fig. 3. Northern blot analysis of OsMADS1 transcript in rice. (A) OsMADS1 expression pattern in rice organs. Total RNAs isolated from leaf (L) and root (R) of two-week-old seedlings, and anther (A), carpel (C), and palea/lemma (P) of anthesis-stage flowers were hybridized with the OsMADS1 probe lacking the MADS domain. Ethidium bromide staining of 25S and 18S rRNAs demonstrates equal amounts of RNA loading. (B) Temporal expression pattern during flower development. Total RNA isolated from rice flowers at different developmental stages was used for detection of OsMADS1 gene expression. 1, young inflorescence (panicle size < 1 cm); 2, young flower (panicle size = 1 to 6 cm); 3, flower at the early vacuolated pollen stage; 4, flower at the late vacuolated pollen stage. Ten mg (samples in Figure A) or 20 mg (samples in Figure B) of total RNA was used.

Ectopic expression of OsMADS1

It was shown previously that ectopic expression of the floral homeotic gene alters floral organ identity in homologous (12, 16, 19, 28) and heterologous systems (14). In order to characterize the functional role of OsMADS1, we have used tobacco plants as a heterologous expression system. The cDNA clone encoding the entire OsMADS1 coding region was placed under the control of cauliflower mosaic virus 35S promoter (3) and transcript 7 terminator using a binary vector pGA748, which is a derivative of pGA643 (1). The chimeric molecule (pGA1209) was transferred to tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) plants

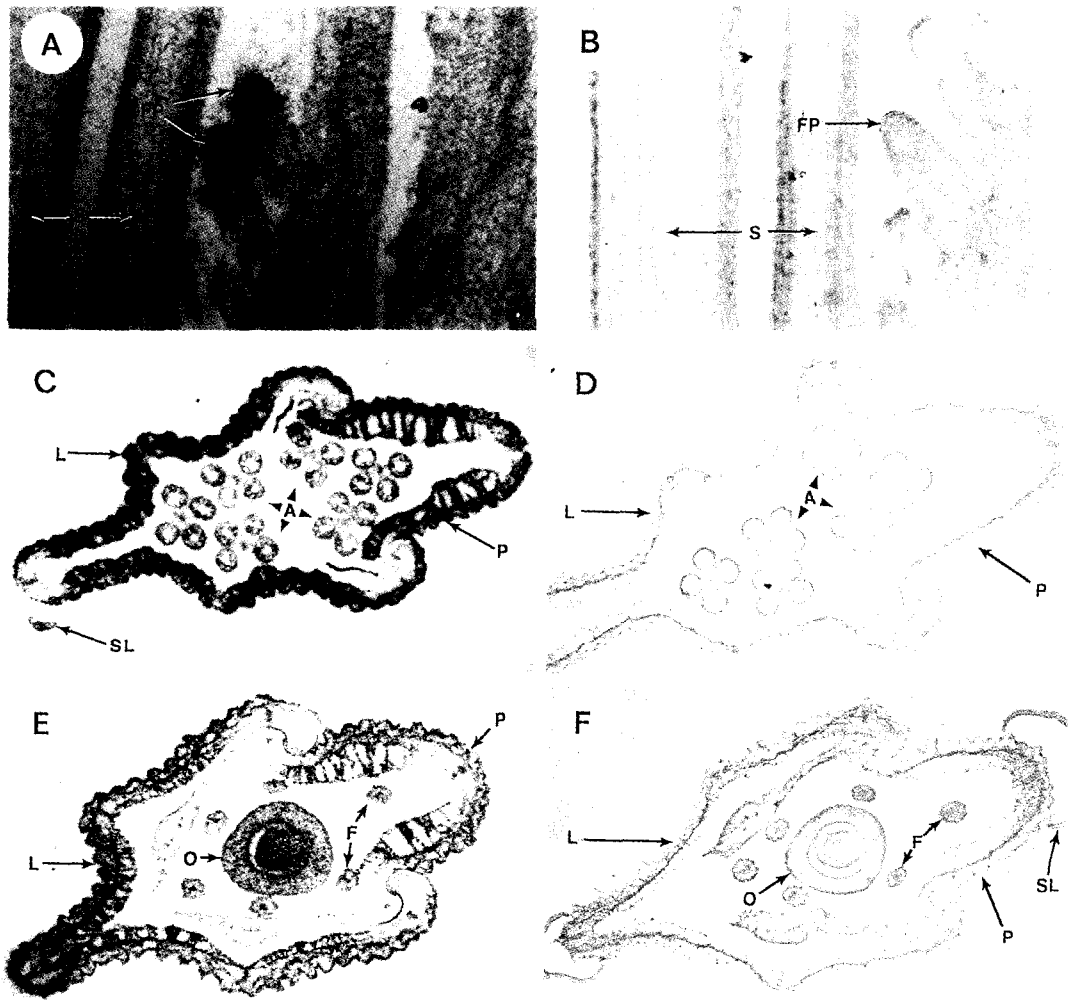

Fig. 4. In situ localization of the OsMADS1 transcript in rice flower. Expression of OsMADS1 RNA was studied by in situ hybridization experiments using longitudinal sections of young inflorescence (A), and cross sections of upper (C) and lower (E) rice flower at late-vacuolated pollen stage. Eight mm sections were hybridized with 35S-labeled antisense RNA which is lacking the MADS box domain (7). The sections were coated with an X-ray emulsion film and exposed for four days. The samples were stained with 0.5% toluidine blue to visualize tissue sections which show negative expression of the gene. Samples B, D, and F are controls. A, anther; F, filament; FP, flower primordia; L, lemma: O, ovary; P, palea; S, sheath; SL, sterile lemma.

using the *Agrobacterium*-mediated Ti plasmid vector system (1). Twenty independent transgenic plants were studied to avoid any artifact. Results showed that most of the primary transgenic plants flowered much earlier compared to the control plants which were transformed with the Ti plasmid vector alone. These plants were significantly shorter and contained several lateral branches (Fig. 5). These phenotypes were inherited to the next generation as a dominant Mendelian trait. Northern blot analysis was conducted on seven

Fig. 5. Phenotypes of transgenic tobacco plants expressing OsMADS1. Transgenic plants were obtained by the cocultivation method (1) using pAL4404 as a disarmed helper-Ti plasmid (9). Left, OsMADS1 transgenic plant #7; right, wild-type SR1 tobacco.

transgenic plants which displayed the early flowering phenotype. The results (Fig. 6) showed that all of the plants accumulated the OsMADS1 transcripts in both vegetative and reproductive organs. Although there were significant differences in gene expression among transgenic plants, the relative expression level was similar between the leaf and flower. Transgenic plant #7, which displayed the most severe symptoms, accumulated the highest level of the transcript. Plants #4, #5, #6, with less severely altered phenotypes, expressed the gene at reduced levels, indicating that the level of OsMADS1 RNA correlated with phenotype. However, progeny from the same parent displayed phenotypic variation. The basis of this variation was investigated with T1 offspring of the transgenic plant #2 in which the transgene segregates as a single locus. OsMADS1 homozygotes were much shorter (34.2×0.8 cm) compared to heterozygotes (51.6×1.4 cm), while the wild-type tobacco plants were 119.8×2.2 cm. The homozygotes flowered two days earlier than the heterozygotes and eight days earlier than the wild type. This indicates that the variation was due to the

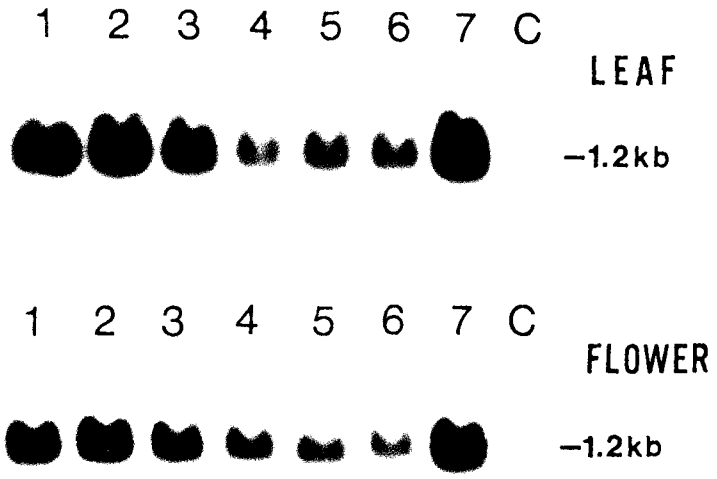

Fig. 6. Northern blot analysis of OsMADS1 transcript in transgenic tobacco. A control plant (C) and seven different transgenic plants (1-7) exhibiting the early flowering and dwarf phenotypes were sampled for preparation of total RNA from leaves and flowers. Twenty mg of total RNA was hybridized with 32P-labeled probe prepared from the OsMADS1 cDNA lacking the MADS domain.

Table 1. Comparison of phenotypes of transgenic plants with non-transformed control. Seeds were collected from selfed fruits of the primary transgenic plants (T0 generation). The seeds were germinated in a peat pellet and grown for two weeks at 16 h light/8 h dark cycles under fluorescent light. These T1 plants were grown under greenhouse conditions. Ten to twenty plants were analyzed for each transgenic line. Standard errors are shown in parentheses. Progenies carrying the transgenes were identified by visually scoring T2 seedlings for kanamycin resistance. The kanamycin sensitive segregants were used as controls (C). Days to flowering include the time from seed germination to the first anthesis. Height and internode length were measured when fruits were fully developed (90 days postgermination).

Transgenic line (#)	Days to flowering	Height (cm)	Internode length (cm)
1	53.0 (2.0)	61.2 (5.8)	5.7 (0.5)
2	54.2 (0.3)	47.6 (1.9)	4.6 (0.2)
3	53.0 (0.4)	64.3 (3.5)	5.8 (0.3)
7	50.6 (0.9)	40.2 (4.4)	3.5 (0.3)
C	61.0 (0.2)	119.8 (2.2)	9.0 (0.3)

gene dosage. Table 1 summarizes characteristics of four independently transformed plants from the T1 generation. Transgenic plants flowered 7 to 10 days earlier than wild-type and their height and internode length appear to be significantly reduced.

Discussion

We report here the isolation and characterization of a rice MADS box gene. The deduced amino acid se-

quence of the rice gene showed a high homology to MADS box proteins, especially in the MADS box domain. The OsMADS1 clone appears to be nearly full length since the estimated transcript length by northern analysis is similar to that of the cDNA clone. The conserved MADS box region is located immediately after the start methionine codon in the rice gene as has been observed in most MADS box genes. Therefore, it is unlikely that the rice clone encodes for a truncated protein. The OsMADS1 sequence is most similar to *AGL2*, AGL4, *AP1* and *SQUA*. The OsMADS1 gene is initially expressed uniformly in young flower primordia and in later developmental stages becomes localized in palea, lemma, and ovary. Vegetative tissues do not show any expression of the gene. The expression pattern of the OsMADS1 gene closely resembled that of *AP1* and *SQUA* (10, 15). Flower specific expression is also common for other MADS box genes (2, 11, 12, 13, 15, 17, 23, 25, 28). Southern analysis revealed that there are at least 10 genes which share a significant homology with the OsMADS1. We have isolated nine independent cDNA clones which contain the conserved MADS box. Detailed characterization of these clones will be reported in a future publication.

We have studied the role of the rice MADS box gene by expressing it in tobacco plants. Ectopic expression of the rice OsMADS1 resulted in early flowering and dwarf phenotypes. It is possible that the rice OsMADS1 product may induce expression of genes which are involved in the induction of flowering. Ectopic expression of OsMADS1 results in accumulation of a protein which may act as a positive regulatory factor similar to *AP1* or *SQUA*. Since the 35S promoter is active in most cell types, the OsMADS1 protein is likely accumulated in shoot meristem and inflorescence meristem where the *AP1* (*SQUA*)-like gene is not activated yet. It was reported earlier that at least two genes, *AP1* and *LEAFY*, are required for the transition of inflorescence meristem into floral meristem in *Arabidopsis* (29). Similarly, *SQUA* and *FLORICAULA* are required for floral organ induction in *Antirrhinum majus* (6). It was determined that *AP1* and *SQUA* belong to the MADS box gene family (6, 29). If AP1 is normally expressed later than *LEAFY*, the ectopic expression of OsMADS1 may bypass the transient period required for normal floral organ development. Alternatively, the protein may interact with a negative factor which normally inhibits flowering. It is also possible that a higher expression of OsMADS1 may enhance the response to flower promoting signals.

Although the exact mechanism by which the gene exerts its effects is not known, we have demonstrated that the OsMADS1 is potentially useful for shortening flowering time and for reducing apical dominance in certain plant species. There is no previous report that other MADS box genes have been used for induction of early flowering and dwarfing. These interesting phenotypes were not apparent in their juvenile state. However, in transgenic plants the axillary bud growth was initiated during the early stage of floral meristem development. The growing shoot apex is known to exert an influence over a range of developmental events including axillary bud growth (26). The effect is most highly observed early in plant development. As plants mature, the emergence of floral organs releases inhibition of the lateral buds and allows them to develop. The active substance responsible for the apical dominance in several plant species has been identified to be a plant growth factor, indoleacetic acid. In the transgenic OsMADS1 plants, the dwarf phenotype may be the result of altered hormonal status due to early flowering.

Early flowering and dwarf phenotypes are important agronomic traits since a balance between vegetative and reproductive growth is a crucial factor that controls crop yields. Enhancement of harvest index in grain crops has been accomplished by the use of dwarfing genes. However, isolation of these genes has been difficult. Moderated expression of the OsMADS1 gene by means of tissue-specific promoters may make it use-

ful as an alternative source of early flowering and dwarfing gene to increase crop productivity.

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