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MADS box genes expressed in developing inflorescences of rice and sorghum

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Abstract With the aim of elucidating the complex genetic system controlling flower morphogenesis in cereals, we have characterized two rice and two sorghum MADS box genes isolated from cDNA libraries made from developing inflorescences. The rice clones *OsMADS24* and *OsMADS45,* which share high homology with the *Arabidopsis AGL2* and *AGL4* MADS box genes, are expressed in the floral meristem, in all the primordia, and in mature floral organs. High expression levels have also been found in developing kernels. The sorghum clone *SbMADS1* is also homologous to *AGL2* and *AGL4*: expression analysis and mapping data suggest that it is the ortholog of *OsMADS24*. The pattern of expression of *SbMADS2*, the other sorghum MADS box gene, suggests that it may play a role as a meristem identity gene, as does *AP1* in *Arabidopsis*, to which it shows considerable homology. The four genes have been mapped on a rice RFLP genetic map: the results are discussed in terms of synteny among cereals.

Key words Flower development · MADS box genes · *Oryza sativa* · *Sorghum bicolor* · Synteny

Introduction

MADS box genes form a large family that is active in a wide range of eukaryotic organisms, ranging from yeast to man and plants (Davies and Schwarz-Sommer 1994; Shore and Sharrocks 1995). They encode putative transcription factors which share a highly conserved domain of 56 amino acids, the MADS box, involved in DNA binding and protein-protein interactions. The MADS box proteins play key roles in several biological processes, all concerning the control of cell fate and differentiation (reviewed in Shore and Sharrocks 1995). In plants, similar functions can be assigned to MADS box proteins, though they seem to be mainly involved in the genetic control of flower development (reviewed in Davies and Schwarz-Sommer 1994).

During the last few years, a model for flower morphogenesis has been proposed, mainly derived from studies on two dicotyledonous model species, *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz 1991; Schwarz-Sommer et al. 1990; Weigel and Meyerowitz 1994). According to this model, several regulating factors interact in a hierarchical manner leading from floral induction to floral organ formation (Ma 1994; Okada and Shimura 1994). Several types of genes are involved in this process; the meristem identity genes determine the transition from inflorescence meristem to floral meristem, while the subsequent activation of organ identity genes in specific cell types and tissues determines the identity of the four floral whorls (sepals, petals, stamens, and carpels). It has been demonstrated that MADS box genes play a very important role in this complex network (for references see Theissen and Saedler 1995). Their importance is also highlighted by the high level of conservation of the MADS box domain. This latter feature has led to the isolation of MADS box genes from several other dicotyledonous species, as well as from more distantly related monocotyledonous species, by means of heterologous hybridization with *Arabidopsis* or *Antirrhinum* probes. This strongly suggests that the regulatory network controlling flower development has been conserved during the evolution of higher plants (Ma 1994; Purugganan et al. 1995; Theissen and Saedler 1995).

In monocots, MADS box genes have been identified in maize (Fischer et al. 1995; Meña et al.1995; Schmidt et al. 1993; Theissen et al. 1995), rice (Chung et al.1994,

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1995; Kang et al. 1995), and orchid (Lu et al. 1993). The fact that MADS box genes also function in monocotyledons is of great interest because of the agronomic importance of the species that belong to this group, not least among which are the cereals. MADS box genes may thus provide a tool for elucidating the complex genetic system controlling flower morphogenesis in cereals.

The structural and functional characterization of new MADS box genes in cereals, however, is required to further our understanding of this complex family of regulatory genes and their involvement in controlling the developmental processes that lead to the formation of reproductive organs and kernels. Hence, we started a program to isolate MADS box genes from cDNA libraries of developing inflorescences from rice and sorghum. Rice was chosen because of its importance as a worldwide crop and, additionally, because of its advantages as a biological model system for cereals in general; our understanding of its genetics is well developed, extremely detailed genetic linkage maps are available (Kurata et al. 1994), and it can be efficiently transformed. Sorghum was chosen because of its agronomical importance and because information regarding the genetic regulation of panicle development is very limited. Although sorghum is closely related to maize, their reproductive strategies differ substantially: maize is monoecious and an outbreeder, while sorghum is mostly an inbreeder with spikelets containing perfect flowers.

Here we report the characterization of two rice and two sorghum MADS box genes, isolated from cDNA libraries of developing inflorescences. Expression analyses indicated that three of them are expressed in the floral meristem and are probably involved in the development of all floral organs, as are *AGL2* and *AGL4* in *Arabidopsis* (Ma et al. 1991), while the fourth gene may represent the sorghum homolog of the *Arabidopsis AP1* gene (Mandel et al. 1992).

Materials and methods

Plant material

Plant tissues for nucleic acid extraction were collected from the rice cultivar Arborio and the sorghum inbred line IS55, grown under normal field conditions. Material was immediately frozen in liquid nitrogen and stored at −80°C until used.

Screening of cDNA libraries

Two cDNA libraries were constructed from poly(A)$^+$ RNA derived from developing rice and sorghum inflorescences of 1–2 cm in length, using the lambda Uni−ZAP XR vector (Stratagene). Approximately 30 000 plaques of each library were screened with a 247/bp fragment containing the MADS box region of the maize clone *ZmOV23* (GenBank U31522; R. Greco unpublished data). The probe was obtained by PCR amplification using the follo wing oligonucleotides 5′-TCGCCAGCATGGTAGAGGAAGGA-3′ and 5′-GCCAGAGGAAGAGCCAACGGTGTG-3′. The probe was labelled using a random priming DNA labelling kit (Boehringer). Pre-hybridization and hybridization were performed overnight at 60° C in 6×SSC, 5× Denhardt's solution, 0.5% SDS, 25 mg/l denatured salmon sperm DNA. Filters were washed twice at 60°C in 1×SSC, 0.1% SDS and once in 0.5×SSC, 0.1% SDS for 20 min each, and then exposed to Kodak XAR film for 24 h. Positive clones were isolated and cDNAs were recovered in pBluescript plasmid vector by in vivo excision, according to the Stratagene protocol.

Sequencing of both strands of each cDNA clone was performed on double-stranded template using a CircumVent thermal dideoxy DNA sequencing kit (New England Biolabs), according to the manufacturer's protocol. Analysis of nucleotide and deduced protein sequences was carried out by using the GCG package.

RNA gel blot analyses

RNA was isolated from both rice and sorghum from roots and shoots of germinated seedlings, from developing inflorescences of approximately 1–4, 8–12, and 16–20 cm in size, from stamens and carpels of mature inflorescences, and from kernels 7 days after pollination. RNA extraction was performed following the protocol proposed by Van Tunen et al. (1988). Total RNA was electrophoresed and blotted onto Hybond N$^+$ filters (Amersham) as described by Ausubel et al. (1987). Aliquots of 20 μg of RNA were loaded in each lane; correct loadings were checked by ethidium bromide staining. Probes (3′-specific) were generated for *OsMADS24* (690 bp, *Bgl*II-*Xho*I fragment), *OsMADS45* (622 bp, *Xho*I fragment), *SbMADS1* (650 bp, *Xho*I fragment), and *SbMADS2* (720 bp, *Pst*I−*Xho*I fragment). Filters were pre-hybridized and hybridized at 65° C in the same solution previously described and then washed to a final stringency of 0.1 × SSC, 0.1% SDS at 65° C.

In situ RNA hybridizations

Developing inflorescences of rice and sorghum, ranging in size from 0.5 mm to 2–3 cm, were collected, fixed, embedded in paraffin, and cut into 8-μm sections as described by Cañas et al. (1994). Plasmids containing the cDNA clones were digested with *Bgl*II (*OsMADS24, OsMADS45,* and *SbMADS1*) or *Pvu*II (*SbMADS2*) to eliminate the MADS box domain. Digoxigenin-labelled antisense RNA probes were generated by in vitro transcription, according to the instructions of Boehringer Mannheim. Hybridization and immunological detection were performed as previously described (Cañas et al. 1994).

RFLP mapping

To map the rice and sorghum MADS box genes isolated, an F_2 population of 192 individuals derived from a cross between rice cultivars Nipponbare and Kasalath was used. DNA extractions from the two parents and from the 192 F_2 plants were performed as described by Kurata et al. (1994). To detect polymorphism, 3′-specific probes were generated for each cDNA clone as previously described and labelled with horseradish peroxidase (HRP) according to the protocol supplied with the ECL Direct Nucleic Acid Labelling and Detection kit (Amersham). Each probe was tested by Southern blot analysis of DNA from parental lines digested with various restriction endonucleases, as described by Kurata et al. (1994). Polymorphism between the two parents was detected when total genomic DNA was restricted with *Bgl*I (*OsMADS24*), *Bgl*II (*OsMADS45* and *SbMADS1*) or *Eco*RI (*SbMADS2*). The 192 individuals of the F_2 population were then typed and segregation analyses were performed using MAPMAKER/EXP 3.0b (Lander et al. 1987).

Results

To identify MADS box genes in rice and sorghum, two cDNA libraries were made from mRNA isolated from

developing inflorescences. About 3×10^4 pfu were screened under low stringency conditions using a probe containing the conserved MADS box region of *ZmOV23*, a carpel-specific clone previously isolated from maize in our laboratory (R. Greco, unpublished data). This approach resulted in the isolation of 18 positive clones from the rice library and 13 from the sorghum library. Two rice clones, *OsMADS24* and *OsMADS45* (*Oryza sativa* MADS) and two sorghum clones, *SbMADS1* and *SbMADS2* (*Sorghum bicolor* MADS) were selected for further characterization.

Sequence analysis

The nucleotide sequences of the four cDNA clones were determined (Fig. 1). All contain a putative ORF, a 3′ untranslated region, and a poly(A) tail. The rice *OsMADS24* and *OsMADS45* are 1016 and 1024 bp in length and code for putative peptides of 237 and 249 amino acids, respectively. The sorghum *SbMADS1* and *SbMADS2* are 1055 and 1186 bp long, respectively, and encode predicted proteins of 231 and 228 amino acids. All the deduced peptides contain the MADS box domain (*OsMADS24* and *SbMADS2* lack a few amino acids at the N-terminus) and the K box dimerization

a

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1    TCGAGAACAAGATCAACAGGCAGGTGACGTTCGCGAAGCGGAGGAATGGGCTGCTCAAGA
       E  N  K  I  N  R  Q  V  T  F  A  K  R  R  N  G  L  L  K  K   20
61   AGGCGTACGAGCTCTCCGTGCTCTGCGACGCCGAGGTCGCCCTCATCATCTTCTCCAACC
       A  Y  E  L  S  V  L  C  D  A  E  V  A  L  I  I  F  S  N  R   40
121  GCGGCAAGCTCTACGAGTTCTGCAGCGGCCAAAGCATGACCAGAACTTTGGAAAGATACC
       G  K  L  Y  E  F  C  S  G  Q  S  M  T  R  T  L  E  R  Y  Q   60
181  AAAAATTCAGTTATGGTGGGCCAGATACTGCAATACAGAACAAGGAAAATGAGTTAGTGC
       K  F  S  Y  G  G  P  D  T  A  I  Q  N  K  E  N  E  L  V  Q   80
241  AAAGCAGCCGCAATGAGTACCTCAAACTGAAGGCACGGGTGGAAAATTTACAGAGGACCC
       S  S  R  N  E  Y  L  K  L  K  A  R  V  E  N  L  Q  R  T  Q  100
301  AAAGGAATCTTCTTGGTGAAGATCTTGGGACACTTGGCATAAAAGAGCTAGAGCAGCTTG
       R  N  L  L  G  E  D  L  G  T  L  G  I  K  E  L  E  Q  L  E  120
361  AGAAACAACTTGATTCATCCTTGAGGCACATTAGATCCACAAGGACACAGCATATGCTTG
       K  Q  L  D  S  S  L  R  H  I  R  S  T  R  T  Q  H  M  L  D  140
421  ATCAGCTCACTGATCTCCAGAGGAGGGAACAAATGTTGTGTGAAGCAAATAAGTGCCTCA
       Q  L  T  D  L  Q  R  R  E  Q  M  L  C  E  A  N  K  C  L  R  160
481  GAAGAAAACTGGAGGAGAGCAACCAGTTGCATGGACAAGTGTGGGAGCACGGCGCCACCC
       R  K  L  E  E  S  N  Q  L  H  G  Q  V  W  E  H  G  A  T  L  180
541  TACTCGGCTACGAGCGGCAGTCGCCTCATGCCGTCCAGCAGGTGCCACCGCACGGTGGCA
       L  G  Y  E  R  Q  S  P  H  A  V  Q  Q  V  P  P  H  G  G  N  200
601  ACGGATTCTTCCATTCCCTGGAAGCTGCCGCCGAGCCCACCTTGCAGATCGGGTTTACTC
       G  F  F  H  S  L  E  A  A  A  E  P  T  L  Q  I  G  F  T  P  220
661  CAGAGCAGATGAACAACTCATGCGTGACTGCCTTCATGCCGACATGGCTACCCTGAACTC
       E  Q  M  N  N  S  C  V  T  A  F  M  P  T  W  L  P  *      237
721  CTGAAGGCCGATGCGACAACCAATAAAAACGGATGTGACGACACAGATCAAGTCGCACCA
781  TTAGATTGATCTTCTCCTACAAGAGTGAGACTAGTAATTCCGTGTTTGTGTGCTAGCGTG
841  TTGAAACTTTTCTGATGTGATGCACGCACTTTTAATTATTATTAAGCGTTCAAGGACTAG
901  TATGTGGTATAAAAGGCCGTACGTGACAGCCTATGGTTATATGCTGCACAAAAACTACGT
961  ATGGTACAGTGCAGTGCCTGTACATTTCATAATTTGCGGTAAAGTTTATTGACTAT
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b

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1    GTGGTGAGATCTAGCTTGGTTGGTTGGTTGCAGCTGGAGATCGATCGGGATGGGGAGGGG
                                                   M  G  R  G    4
61   TCGGGTGGAGCTGAAGAGGATCGAGAACAAGATCAACCGGCAGGTGACGTTCGCCAAGCG
       R  V  E  L  K  R  I  E  N  K  I  N  R  Q  V  T  F  A  K  R   24
121  CAGGAATGGCCTGCTCAAGAAGGCGTACGAGCTCTCCGTCCTCTGCGACGCCGAGGTCGC
       R  N  G  L  L  K  K  A  Y  E  L  S  V  L  C  D  A  E  V  A   44
181  CCTCATCATCTTCTCCAACCGCGGCAAGCTCTACGAGTTCTGCAGCACCCAGAGCATGAC
       L  I  I  F  S  N  R  G  K  L  Y  E  F  C  S  T  Q  S  M  T   64
241  TAAAACGCTTGAGAAGTATCAGAAATGCAGTTACGCAGGACCCGAAACAGCTGTCCAAAA
       K  T  L  E  K  Y  Q  K  C  S  Y  A  G  P  E  T  A  V  Q  N   84
301  TAGAGAAAGTGAGCAATTGAAAGCTAGCCGCAATGAATACCTCAAACTGAAGGCAAGGGT
       R  E  S  E  Q  L  K  A  S  R  N  E  Y  L  K  L  K  A  R  V  104
361  TGAAAATTTACAACGGACTCAAAGAAATTTGCTGGGTGAAGATCTTGATTCATTAGGCAT
       E  N  L  Q  R  T  Q  R  N  L  L  G  E  D  L  D  S  L  G  I  124
421  AAAAGAGCTCGAGAGCCTAGAGAAGCAGCTTGATTCATCCCTGAAGCACGTCAGAACTAC
       K  E  L  E  S  L  E  K  Q  L  D  S  S  L  K  H  V  R  T  T  144
481  AAGGACAAAACATCTGGTTGACCAACTGACGGAGCTTCAGAGAAAGGAACAAATGGTTTC
       R  T  K  H  L  V  D  Q  L  T  E  L  Q  R  K  E  Q  M  V  S  164
541  TGAAGCAAATAGATGCCTTAGGAGAAAACTGGAGGAAAGCAACCATGTTCGCGGGCAGCA
       E  A  N  R  C  L  R  R  K  L  E  E  S  N  H  V  R  G  Q  Q  184
601  AGTGTGGGAGCAGGGCTGCAACTTAATTGGCTATGAACGTCAGCCTGAAGTGCAGCAGCC
       V  W  E  Q  G  C  N  L  I  G  Y  E  R  Q  P  E  V  Q  Q  P  204
661  TCTTCACGGCGGCAATGGGTTCTTCCATCCACTTGATGCTGCTGGTGAACCCACCCTTCA
       L  H  G  G  N  G  F  F  H  P  L  D  A  A  G  E  P  T  L  Q  224
721  GATTGGGTACCCTGCAGAGCATCATGAGGCGATGAACAGTGCGTGCATGAACACCTACAT
       I  G  Y  P  A  E  H  H  E  A  M  N  S  A  C  M  N  T  Y  M  244
781  GCCCCCATGGCTACCATGATGATGACGGGGACAATGAATTACGAAATAACAAGGATATGT
       P  P  W  L  P  *                                        249
841  GGCATATATGTGGTGCCGCATACATGCATGTATCATGGCTAGCTACTTAATTGGAGTGAT
901  GGATTTGAACTAGTTTCGTATGTAGCCTGTTTGTGTGTAACTTGTGTGAGATACTACCTT
961  AAAAACTATCGGTGTCTGTTGAACATATTCTGCGATCAACTTTAAGCGTATTATTATCAT
1021 TTTC
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c

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1    GCTGAGGGTAGCTGCAGCTGGGGCTAGCTGGAAGGGTTGGGAGCCATGGGGAGGGGGCGG
                                                   M  G  R  G  R    5
61   GTTGAGCTGAAGCGGATCGAGAACAAGATCAACCGCCAGGTCACCTTCGCCAAGCGCCGC
       V  E  L  K  R  I  E  N  K  I  N  R  Q  V  T  F  A  K  R  R   25
121  AACGGGCTGCTCAAGAAGGCGTACGAGCTCTCCGTGCTCTGCGACGCCGAGGTCGCGCTC
       N  G  L  L  K  K  A  Y  E  L  S  V  L  C  D  A  E  V  A  L   45
181  ATCATCTTCTCCAACCGCGGCAAGCTCTACGAGTTCTGCAGCGGACAGAGCATCACAAAA
       I  I  F  S  N  R  G  K  L  Y  E  F  C  S  G  Q  S  I  T  K   65
241  ACACTTGAGAGGTACGAAAAGCATATGAGGCCAGATACTGCTGTACAGAACAAGGAGAAC
       T  L  E  R  Y  E  K  H  M  R  P  D  T  A  V  Q  N  K  E  N   85
301  GAGTTAGTCCAGAGCAGTCGGAATGAGTACCTTAAACTGAAAGCAAGGGTGGATAATTTA
       E  L  V  Q  S  S  R  N  E  Y  L  K  L  K  A  R  V  D  N  L  105
361  CAAAGGACTCAGAGGAATTTGCTTGGTGAAGATCTGGGGTCACTTGGTATCAAAGAGCTT
       Q  R  T  Q  R  N  L  L  G  E  D  L  G  S  L  G  I  K  E  L  125
421  GAGCAGCTCGAGAAGCAACTTGATTCATCCTTAAGGCACATAAGATCCACAAGGACACAA
       E  Q  L  E  K  Q  L  D  S  S  L  R  H  I  R  S  T  R  T  Q  145
481  CATATGCTTGATCAGCTCACTGATCTTCAGAGGAGGGAGCAAATGCTGTGTGAAGCAAAT
       H  M  L  D  Q  L  T  D  L  Q  R  R  E  Q  M  L  C  E  A  N  165
541  AAGTGCCTTAGAAGAAAGCTGGAGGAGACCAGCAACCAGGTGCATAGCAAGTGTGGAGCA
       K  C  L  R  R  K  L  E  E  T  S  N  Q  V  H  S  K  C  G  A  185
601  CGGTGCCAACTTACTCGGCTACGAGCGGCCACTCCCCCCCACAGCAGGCCCCATCACATA
       R  C  Q  L  T  R  L  R  A  A  T  P  P  H  S  R  P  H  H  I  205
661  TTGGCAATGGATTGTTCTTTCATCCCCTGGAAGCTGCAGCAGAGCCAACCCTGCAGATCG
       L  A  M  D  C  S  F  I  P  W  K  L  Q  Q  S  Q  P  C  R  S  225
721  GGTTCGCTCCTGAACATATGAATAACTTCATGCCGACATGGCTACCCTGAAGATGGATCT
       G  S  L  L  N  I  *
781  TTGAGGCAAAGGAATAAATAAAGATGCGATGGCCAGCATCATCTACTACTGCAAGAGTGA
841  CAGTCATATATATATATATATATGACCTAGTTTGTGTGTCGATGTGTCGAAACGCATAAA
901  TTTGTGATTTGTTATATGTAATGTATAAGCACAAACGAAACATGCAGGACTAGTTGCTTG
961  CTTCGATGTATTTGTGTGTATCCGTGTAAAGGGCCGTGCGCACAGCATATGAGTGATGAG
1021 ATTTTCTCTAATAAGGCCGTTATGCCGCCAATCGT
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d

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1    GCAAGGTGCAGCTCAAGCGGATAGAGAACAAGATAAACCGGCAGGTGACCTTCTCCAAGC
       K  V  Q  L  K  R  I  E  N  K  I  N  R  Q  V  T  F  S  K  R   20
61   GCCGCAACGGGCTGCTCAAGAAGGCGCACGAGATCTCCGTCCTCTGCGACGCCGAGGTCG
       R  N  G  L  L  K  K  A  H  E  I  S  V  L  C  D  A  E  V  A   40
121  CCGTCATCGTCTTCTCCCCCAAGGGCAAGCTCTATGAGTACGCCACCGACTCCCGCATGG
       V  I  V  F  S  P  K  G  K  L  Y  E  Y  A  T  D  S  R  M  D   60
181  ACAAAATTCTCGAACGTTATGAGCGATATTCCTATGCTGAAAAGGCTGTTATTTCAGCTG
       K  I  L  E  R  Y  E  R  Y  S  Y  A  E  K  A  L  I  S  A  E   80
241  AATCTGAAAGTGAGGGAAACTGGTGCCACGAATACAGGAAACTGAAGGCCAAAATTGAGA
       S  E  S  E  G  N  W  C  H  E  Y  R  K  L  K  A  K  I  E  T  100
301  CCATTCAAAAATGCCACAAGCACCTGATGGGAGAGGATCTAGAGTCTTTGAATCCCAAAG
       I  Q  K  C  H  K  H  L  M  G  E  D  L  E  S  L  N  P  K  E  120
361  AGCTCCAACAACTAGAGCAGCAGCTGGAGAGCTCACTGAAGCACATCAGATCAAGAAAGA
       L  Q  Q  L  E  Q  Q  L  E  S  S  L  K  H  I  R  S  R  K  S  140
421  GCCACCTTATGGCTGAGTCTATTTCTGAACTACAGAAGAAGGAGAGGTCACTGCAGGAGG
       H  L  M  A  E  S  I  S  E  L  Q  K  K  E  R  S  L  Q  E  E  160
481  AGAACAAGGCTCTACAGAAGGAACTTGCGGAGAGGCAGAAGGCGGCCGCGAGCAGGCAGC
       N  K  A  L  Q  K  E  L  A  E  R  Q  K  A  A  A  S  R  Q  Q  180
541  AGCAGCAAGGTGCAGTGGGACCAGCAGACACAGACCCAGGCCCAGACAAGCTCATCATCG
       Q  Q  G  A  V  G  P  A  D  T  D  P  G  P  D  K  L  I  I  V  200
601  TCCTCCTTCATGATGAGGCAGGATCAGCAGGGTCTGCCGCCTCCACAAAACATATGCTTC
       L  L  H  D  E  A  G  S  A  G  S  A  A  S  T  K  H  M  L  P  220
661  CCGCCGCTGATAATCGGAGAGAGAGGTGAAGAGGTGGCTGCGGCGGCGCAGCAGCAGCAG
       A  A  D  N  R  R  E  R  *
721  CCACCACCAGGGCAGGCGCAGCAAGCGCAGTCCGCATCGCAGGTCTGCCGCCATGGA
781  TGCTGAGCCACCTCAATGCATAAGCAGAGGGCGATAGCAAATCGACCTCTCTCGTCATGA
841  ACCATGACGTACGATTACCATATGGTTGCCGTGCCTCTCCCATCGAATGGCGAGCAATGG
901  CACGCTCCTGCAAGTGATTGCTCGATCACCGATGATTAAAACCCTAGCGATGGCAGCAGC
961  AATTAAGCTAAACTATTGTTATGTTTGCAAGAAAGGGTAACCCGCTGTGTAATCTTTGTC
1021 TCCAGCTAGCATGTACGTACCAACTGAGATGCATGCATGCGTATATCAGCATATGCTTTA
1081 TTACTGCTCAGTTACCCTTGAATCTAGCAGTGCTTTTGGTGAGAGGGTGCAGTTTACTTT
1141 AAACATATGGGTTTGTGACTGTAAATAGTATTTTTAACTAGGAGTT
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Fig. 1 Nucleotide and deduced amino acid sequences of the cDNA clones *OsMADS24* (**a**), *OsMADS45* (**b**), *SbMADS1* (**c**), and *SbMADS2* (**d**). MADS box and K box are *underlined.* The putative nuclear localization signal is in *bold*; the putative phosphorylation site is *double underlined.* Nucleotide and amino acid positions are shown on the *left* and *right margins*, respectively. **a** and **d** represent incomplete cDNAs. The sequences have been submitted to the EMBL Data Library and assigned the following accession numbers: U32109 (*OsMADS24* U31994 (*OsMADS45*); U49734 (*S6MADS1*); U32110 (*SbMADS2*)

Table 1 Sequence comparison between the deduced peptides encoded by *OsMADS24, OsMADS45, SbMADS1*, and *SbMADS2*. Percentages of overall amino acid identity are indicated; between *brackets*, are shown the identities in the MADS box domain (*left*) and in the downstream region (*right*)

	OsMADS45	SbMADS1	SbMADS2
OsMADS24	78.2 (100–72.9)	75.3 (100–68.8)	44.1 (83–33.9)
OsMADS45	–	68.9 (100–58.5)	47.3 (82–37.3)
SbMADS1	–	–	44.3 (81–32.7)

domain. In addition, a putative nuclear localization signal (KRIENNTSRQVTFCKRR; Robbins et al. 1991) and a potential calmodulin-dependent phosphorylation site (RXX[S/T]; Cohen 1988) are present within the MADS box and are highly conserved. Downstream of the K box, all peptides share a Q-rich region which might represent a potential activation domain (Mitchell and Tjian 1989). All these features are in accordance with the presumed role for these gene products as transcription factors (Schwarz-Sommer et al. 1990).

Alignment of the deduced amino acid sequences revealed a high level of sequence similarity between *OsMADS24, OsMADS45*, and *SbMADS1*, while *SbMADS2* showed a lower degree of homology with the other sequences both inside and outside the MADS box domain. Table 1 shows the result of the comparisons.

Database comparisons of the predicted *OsMADS24, OsMADS45,* and *SbMADS1* proteins showed the highest homologies (about 60% amino acid identity) with AGL2 and AGL4 of *Arabidopsis* (Ma et al. 1991). The MADS box domains are almost identical, while the percentage identity considering only the MADS and the K box regions is over 70% (Table 2, Fig. 2a). By comparison, the deduced *SbMADS2* peptide appeared to be most similar to AP1 of *Arabidopsis* (Mandel et al.1992): the two share 56% sequence identity over the whole length, 89% in the MADS domains and 69% in the first 170 amino acids, including the K box region (Table 2). Recently, a putative maize ortholog of *AP1* has been cloned by Meña et al. (1995), namely *ZAP1.* Sequence comparison between the deduced peptides encoded by *ZAP1* and *SbMADS2* show an overall amino acid identity of 83%: the MADS box domains are identical and only two substitutions are present in the first 170 amino acids. The alignment between SbMADS2, AP1, and ZAP1 is shown in Fig. 2b.

Table 2 Database comparison of the deduced peptides OsMADS24, OsMADS45, SbMADS1, and SbMADS2. Percentages of overall amino acid identity are indicated; between *brackets*, are shown the identities in the MADS box domain (*left*) and in the first 170 amino acids (MADS and K box domains; *right*)

	AGL2	AGL4	AP1	ZAP1
OsMADS24	59.3 (100–74.1)	59.2 (95.6–72.8)	–	–
OsMADS45	62.2 (100–74.7)	59.9 (96.5–74.7)	–	–
SbMADS1	57.6 (100–73.4)	57.2 (96.5–72.1)	–	–
SbMADS2	–	–	56.0 (88.6–68.6)	82.9 (100–98.9)

Northern blot analysis

The expression pattern of the four MADS box genes was first characterized by northern blot analysis using total RNA isolated from different tissues of rice and sorghum. To avoid cross-hybridization, each RNA gel blot was probed with 3′ terminal fragment of the corresponding cDNA clone lacking the MADS box region.

The rice and sorghum genes are exclusively expressed in flowers and no signal was present in vegetative tissues such as roots or shoots. In the various rice floral tissues, the *OsMADS24* transcript was detected in immature inflorescences at different stages of development, in mature stamens and carpels, and in developing kernels (Fig. 3). *OsMADS45* showed a pattern of expression similar to that of *OsMADS24* (data not shown).

Northern blot hybridization of the sorghum gene *SbMADS1*, revealed a pattern of expression restricted to developing inflorescences, mature reproductive organs, and developing kernels, as observed for the rice genes (data not shown). With regard to *SbMADS2*, transcripts were only detectable in developing sorghum inflorescences: no expression was found in mature stamens and carpels nor in developing kernels. Moreover, the strength of the signal in the inflorescences seemed to decrease from younger to later stages of development (Fig. 4).

In situ hybridization

In order to analyze the expression pattern of *OsMADS24*, *OsMADS45, SbMADS1*, and *SbMADS2* during early developmental stages of the rice and sorghum spikelets, in situ hybridization experiments were performed on inflorescences at various stages of maturation. Digoxigenin-labelled antisense RNA probes corresponding to the 3′ end of the cDNA clones were used.

Schematic diagrams of rice and sorghum flowers are shown in Fig. 5. The main differences concern the number of stamens, higher in rice, and the presence of larger empty glumes in sorghum. Both flowers lack the perianth: instead of sepals and petals there are two small organs, called lodicules. The apical meristem of the spikelet primordium starts the differentiation by generating successively the rudimentary glumes, the empty glumes, the lemma, the palea, a pair of lodicules, and the reproductive organs, the stamens and the pistil (Takeoka et al. 1993).

OsMADS45 transcripts are detectable in the rice spikelet primordium (Fig. 6a). Later in development,

Fig. 2a, b Sequence comparison between predicted MADS box proteins. **a** Alignment between the deduced peptides OsMADS24, OsMADS45, and SbMADS1 with AGL2 and AGL4 of *Arabidopsis*. **b** Alignment between SbMADS2, AP1 of *Arabidopsis*, and ZAP1 of maize. MADS box and K box are *underlined. Asterisks* indicate amino acid identity, while *dashes* indicate gaps introduced to maximize alignments

a

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AGL2      MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSSSNMLKTLDRYQKCSYGSIE
AGL4      ****************************************S**V***********T*******E**********
OsMADS24  ----------**********************************************GQS*TR**E****F***GPD
OsMADS45  ************************************************************TQS*T***EK******AGP*
SbMADS1   ************************************************************GQSIT***E**E*--HMRPD

--VNNKPAKELENSYREYLKLKGRYENLQROORNLLGEDLGPLNSKELEQLERQLDGSLKQVRSIKTQYMLDQLSDLQNKEQMLLETN
--**********************************************************C**************G**HI**DA*
TAIQ**ENELVQS*RN******A*V*****T**********T*GI*******K***S**RHI**TR**H*****T***RR****C*A*
TA*Q*RESEQ*KA*RN******A*V*****T*********DS*GI****S**K***S***H**TTR*KHLV***TE**R****VS*A*
TA*Q**ENELVQS*RN******A*VD****T**********S*GI*******K***S**RHI**TR**H*****T***RR****C*A*

RALAMKLDD----MIGVRSHHM-GGWEGGE-Q--NVTYAHHQAQSQGLYQPLEC--NPTLQMGYDNPVCSEQITATTQAQAQPGNGYI
***S***E*----*****H**IG******DQ*---*IA*G*P**H******S****--D****I**SH******MAV*V*G*S*Q*****
KC*RR**EE-SNQLH*-QVWEHGATLL*Y*R*SPHAVQQVPPHGGN*FFHS**AAAE****I*F-----------*PEQ---MN*SCV
*C*RR**EE-SNHVR*QQVWEQGCNLI*Y*R*P--EVQQ-PLHGGN*FFH**DAAGE****I*Y----------PAEHHEAMNSACM
KC*RR**EETSNQVHSK---------C*ARC*LTRLRAATPPHSRPHHILAMD*SF-----IPW----------KLQQSQPCRSGSL

PGWML----
*****----
TAF*PTWLP
NTY*PPWLP
LNI------
```

b

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AP1       MGRGRVQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALVVFSHKGKLFEYSTDSCMEKILERYERYSYAERQ
SbMADS2   ----K******************N****************VI***P****Y**A***R*D************K*
ZAP1      ****K******************N****************VI***P****Y**A***R*D************K*

LIAPESDVNTNWSMEYNRLKAKIELLERNQRHYLGEDLQAMSPKELQNLEQQLDTALKHIRTRKNQLMYESINELQKKEKAIQEQNSM
**SA**ESEG**CH**RK******TIQKCHK*LM****ESLN*****Q*****ESS*****S**SH**A***S******RSL**E*KA
**SA**ESEG**CH**RK******TIQKCHK*LM****ESLN*****Q******SS*****S**SH**A***S******RSL**E*KA

LSKQIKEREKILRAQQEQ------WDQQNQGHNMPPP------LPPQQHQIQHPYMLSHQPSPF------LNMGGLYQEDDPMAMRND
*Q*ELA**QKAAASR*Q*Q------GAVGPADTD*G*DKLIIV*LHDEAG----------SA*SAASTKHMLPAADN
*Q*ELA**QKAVASR*Q*QQQQVQ****THAQAQTSSSSSSFMMRQD*QGLPP*HNICFP*LTMGDRGEE*AAAAAA*QQQ*LPGQAQ

LELTLEPVYNCNLGCFAA
RRER--------------
PQ*RIAGLPPWMLSHLNA
```

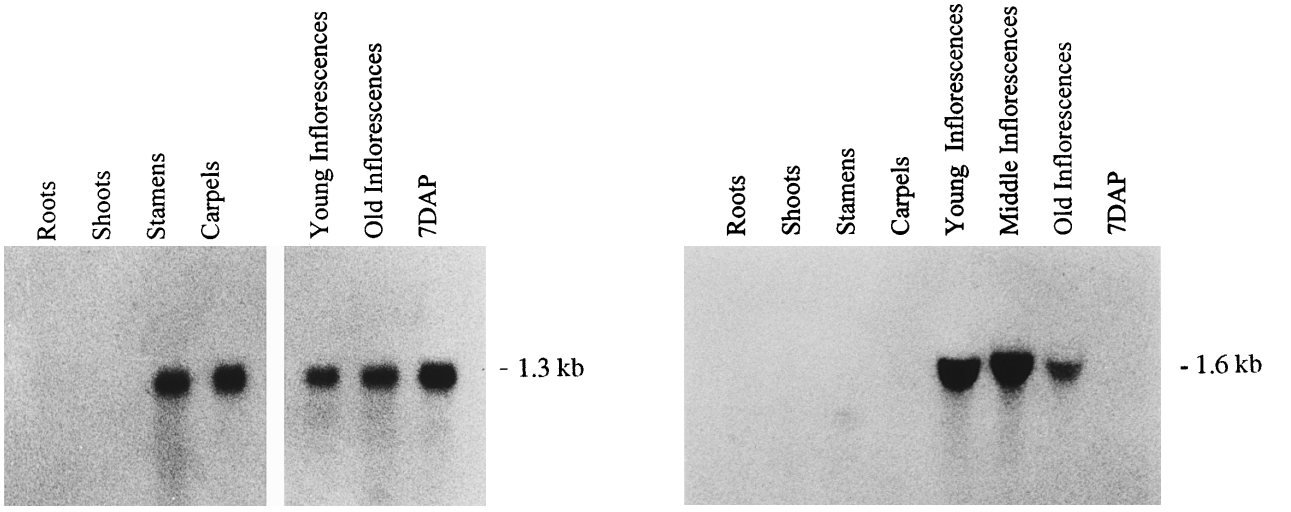

Fig. 3 Northern blot analysis of *OsMADS24*. Total RNA, (20 μg) isolated from various non-reproductive and reproductive tissues of rice, was loaded in each lane. The blot was probed with the specific 3′ end of the *OsMADS24* cDNA. Young and old inflorescences indicate RNA extracted from developing inflorescences recovered from rice panicles of 1–4 and 16–20 cm in size, respectively

Fig. 4 Northern blot analysis of *SbMADS2*. Total RNA (20 μg), isolated from various non-reproductive and reproductive tissues of sorghum, was loaded in each lane. The blot was probed with the specific 3′ end of the *SbMADS2* cDNA. Young, middle and old inflorescences indicate RNA extracted from developing inflorescences recovered from sorghum panicles of 1–4, 8–12, and 16–20 cm in size, respectively

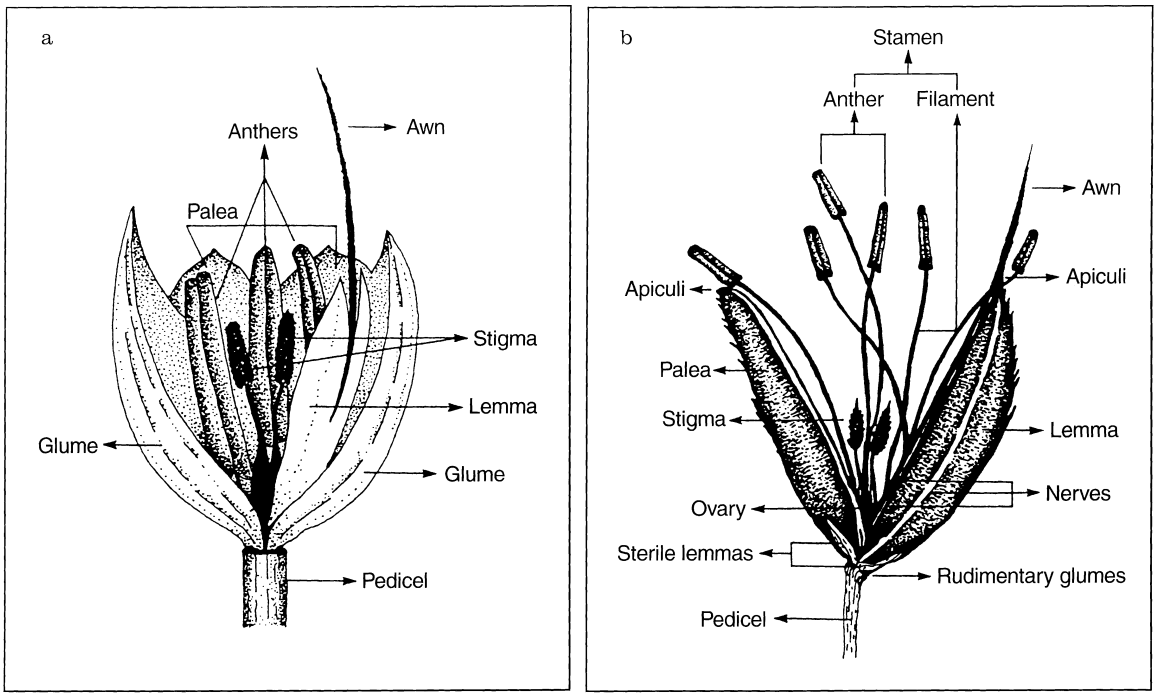

Fig. 5 Structure of the rice (**a**) and sorghum (**b**) flower

when stamens are differentiating and before they become enclosed by the lemma and the palea, the expression of *OsMADS45* appears to be localized specifically and abundantly in lodicules, developing stamens, and the pistil primordium (Fig. 6b). This pattern of expression is maintained through the young spikelet stage, when young stamens have just differentiated into anthers and filaments and ovary and style are formed in the young pistil (Fig. 6c). No expression was detected in empty glumes nor in lemma and palea at any stage of development. *OsMADS24* showed the same in situ expression pattern as *OsMADS45,* and a similar picture was found for *SbMADS1* during sorghum spikelet development (data not shown).

The other sorghum gene, *SbMADS2,* starts to be expressed very early in sorghum floral meristem (Fig. 6d,e) where the primordia of the floral organs will arise. However, no expression signal is detected when the floral organs undergo the final stages of maturation (Fig. 6f).

RFLP mapping

In order to localize *OsMADS24* and *OsMADS45* on the rice genome, we performed a restriction fragment length polymorphism (RFLP) analysis on a F_2 population derived from a cross between rice cultivars Nipponbare and Kasalath (Kurata et al. 1994). The 3′ ends of the clones, lacking the MADS box region, were used as probes. Single band patterns were obtained in Southern blot experiments (data not shown), allowing the mapping of each gene to a single locus. Using the ECL-labelled 3′ fragments of the two rice cDNA clones, 186 F_2 plants were typed and the segregation data were analyzed with MAPMAKER/EXP 3.0b (Lander et al. 1987).*OsMADS24* was placed on chromosome 9 between molecular markers R2840 and C1263, while *OsMADS45* cosegregated with W152 on chromosome 8 of the most densely saturated rice RFLP linkage map produced at the Rice Genome Project in Tsukuba, Japan (Kurata et al. 1994; M. Yano, personal communication).

The sorghum clones were also mapped on the rice genetic map by heterologous hybridization of their 3′ terminal fragments to the F_2 population previously described. These experiments revealed cosegregation of *SbMADS1* with rice *OsMADS24*, while *SbMADS2* cosegregated with the molecular marker W112 on chromosome 7.

Discussion

The MADS box genes of monocotyledons have so far, been investigated mainly in maize but also in orchid and rice. In the latter species, four MADS box genes have been described to date, one being the ortholog of *AGAMOUS* (Kang et al. 1995), two being homologous to *GLOBOSA* (Chung et al. 1995), and the fourth being involved in floral induction (Chung et al. 1994). In contrast, no studies concerning MADS box genes in sorghum have been reported till now. Screening of two cDNA libraries from developing inflorescences of rice and sorghum with a probe containing the MADS box region of a maize MADS box gene has resulted in the isolation of several cDNA clones from rice and from sorghum. In this work, we characterized two MADS box genes active in rice inflorescences and we also report the

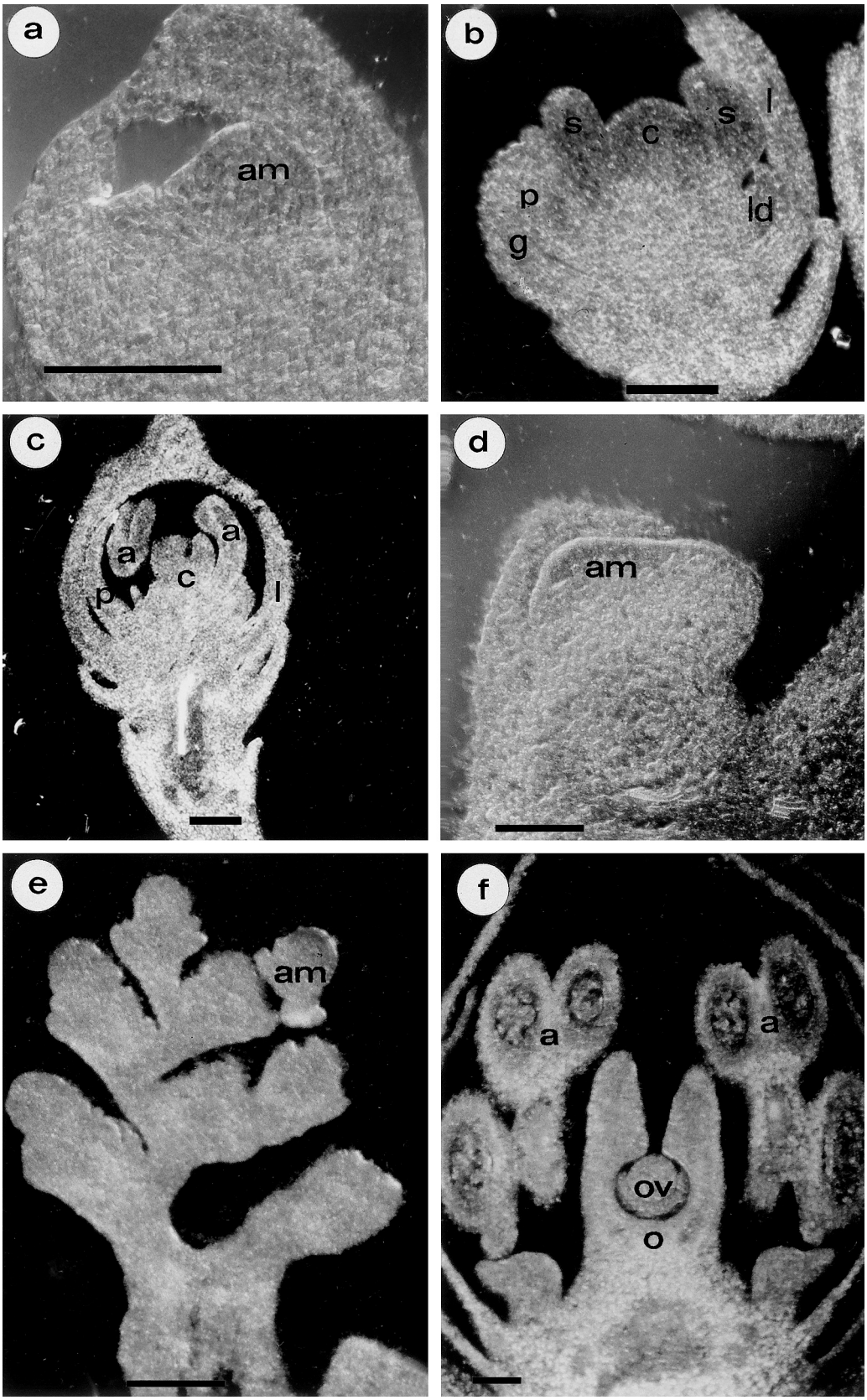

Fig. 6a–f In situ localization of *OsMADS45* mRNA in developing inflorescences of rice and of *SbMADS2* mRNA in developing inflorescences of sorghum. **a** Bright-field micrographs of a rice spikelet primordium. **b, c** Dark-field micrographs of a young rice spikelet. **d, e** Dark-field micrographs of a sorghum spikelet primordium. **f** Dark-field micrograph of a sorghum spikelet with mature anthers and carpel. (*am* Apical meristem, *g* glumes, *l* lemma, *p* palea, *ld* lodicules, *s* stamens, *c* carpel, *a* anthers, *o* ovary, *ov* ovule) *Bars* 0.5 mm

identification of the first MADS box genes putatively involved in sorghum flower development.

Rice MADS box genes

We have identified two MADS box genes in rice, *OsMADS24* and *OsMADS45*, the putative products of which show extensive similarity to AGL2 and AGL4 of *Arabidopsis* (Ma et al. 1991). In situ hybridization experiments have previously shown that *AGL2* and *AGL4* start to be expressed early in flower development, when the floral meristem has just been established but before the organ primordia have emerged. High expression level was also found for *AGL2* in developing embryos and seed coats (Flanagan and Ma 1994; Savidge et al. 1995). It has therefore been proposed that these genes might play a role in the development of all floral organs, most probably by acting as intermediates between the meristem identity and organ identity genes (Savidge et al. 1995). In addition, they might also be involved in seed and embryo development.

The expression pattern of *OsMADS24* and *OsMADS45*, analyzed first by northern blot hybridization to RNA isolated from various rice tissues, revealed specificity to floral organs and developing kernels. Furthermore, in situ hybridizations on inflorescences at different developmental stages demonstrated that both rice genes were specifically expressed in the floral meristem and in the primordia of all the floral organs, which is in agreement with the expression pattern of *AGL2* and *AGL4*. These observations suggest that *OsMADS24* and *OsMADS45* may play similar roles in rice flower development to those of *AGL2* and *AGL4* in *Arabidopsis*, though for neither the rice nor the *Arabidopsis* genes has a function yet been determined.

OsMADS24 and *OsMADS45* were mapped by RFLP analysis on two different chromosomes by using the most densely saturated genetic linkage map of rice available (Kurata et al. 1994; M. Yano, personal communication). However, this map has not yet been integrated with the classical linkage maps, therefore it was not possible to correlate the map positions of our clones with any known rice mutant involved in the control of flower development and/or plant fertility.

Sorghum MADS box genes

SbMADS1 and *SbMADS2* are the two MADS box genes whose cDNAs have been isolated from sorghum. Sequence comparison and expression pattern analysis performed with *SbMADS1* suggest that it is the ortholog of one of the rice genes previously described. *SbMADS2* belongs to a different class of MADS box genes and shows limited homology with *SbMADS1* and the rice clones. The deduced peptide sequence of SbMADS2 revealed considerable similarity to AP1 of *Arabidopsis*, which is known to specify the identity of the floral meristem and to determine sepal and petal development (Gustafson-Brown et al. 1994; Mandel and Yanofsky 1995; Mandel et al. 1992). *SbMADS2* was found to be expressed very early in the floral meristem, as was also observed for *AP1* of *Arabidopsis*. *AP1* is expressed in the two outer floral whorls during organogenesis, whereas *SbMADS2* expression is abolished in the whole flower at late developmental stages. However, we have to consider that the sorghum flower lacks sepals and petals. The cloning of the maize ortholog of *AP1* (*ZAP1,* Meña et al. 1995), to which *SbMADS2* shows extensive sequence similarity, indicates the presence of *AP1* homologs in cereals, though the conservation of *AP1* function is not yet proven.

A segregating sorghum population typed for molecular markers was not available, therefore we were not able to locate the sorghum genes on a sorghum genetic linkage map. However, taking advantage of the cross-hybridization with rice DNA, we placed both sorghum MADS box genes on rice genetic linkage groups (Kurata et al. 1994; M. Yano, personal communication). The probe derived from *SbMADS1* identified the same locus as *OsMADS24*, reinforcing the hypothesis that these genes might be orthologs. More interestingly, *SbMADS2*, which belongs to another class of MADS box genes, identified a new locus that could possibly represent the rice homolog of the *AP1* and *ZAP1* genes. The mapping of these genes is particularly relevant, especially in terms of the extensive colinearity observed between the rice genome and genomes of other cereals, among which are sorghum and maize (Ahn and Tanksley 1993; Moore et al. 1995). Many MADS box genes from maize have already been mapped (Fischer et al. 1995; Meña et al. 1995; Schmidt et al. 1993; Theissen et al. 1995) and have been found to be scattered throughout the maize genome. It is therefore worth noting that *SbMADS2* maps to a region of rice chromosome 7 which is syntenic to maize chromosomes 2L and 7S where *ZAP1a* and *ZAP1b* were, respectively, placed (Meña et al. 1995). A similar syntenic relationship has been found between *OsMADS24*, placed on rice chromosome 9, and *ZMM7*, a member of the maize MADS box gene family, mapped on maize chromosome 7 (Fischer et al. 1995). Furthermore, the *OsMADS24* MADS domain sequence is identical to the partial one available for *ZMM7*. Taken together, these observations represent interesting clues to the existence of a maize ortholog of *OsMADS24*, possibly already identified in *ZMM7*. Being a very complex gene family, MADS box genes could thus be very useful for clarifying syntenic relationships between cereals and elucidating both cereal genome evolution and MADS box gene diversification.

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