Functional Characterization of *OsMADS18*, a Member of the *AP1/SQUA* Subfamily of MADS Box Genes[1][w]

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MADS box transcription factors controlling flower development have been isolated and studied in a wide variety of organisms. These studies have shown that homologous MADS box genes from different species often have similar functions. *OsMADS18* from rice (*Oryza sativa*) belongs to the phylogenetically defined *AP1/SQUA* group. The MADS box genes of this group have functions in plant development, like controlling the transition from vegetative to reproductive growth, determination of floral organ identity, and regulation of fruit maturation. In this paper we report the functional analysis of *OsMADS18*. This rice MADS box gene is widely expressed in rice with its transcripts accumulated to higher levels in meristems. Overexpression of *OsMADS18* in rice induced early flowering, and detailed histological analysis revealed that the formation of axillary shoot meristems was accelerated. Silencing of *OsMADS18* using an RNA interference approach did not result in any visible phenotypic alteration, indicating that *OsMADS18* is probably redundant with other MADS box transcription factors. Surprisingly, overexpression of *OsMADS18* in Arabidopsis caused a phenotype closely resembling the *ap1* mutant. We show that the *ap1* phenotype is not caused by down-regulation of *AP1* expression. Yeast two-hybrid experiments showed that some of the natural partners of AP1 interact with OsMADS18, suggesting that the *OsMADS18* overexpression phenotype in Arabidopsis is likely to be due to the subtraction of AP1 partners from active transcription complexes. Thus, when compared to AP1, OsMADS18 during evolution seems to have conserved the mechanistic properties of protein-protein interactions, although it cannot complement the AP1 function.

Compared to dicot species such as Arabidopsis, *Antirrhinum majus*, or *Petunia hybrida*, there is much less known about genes controlling flower development in monocots, such as rice (*Oryza sativa*) and maize (*Zea mays*). More than a decade of extensive studies of floral developmental mutants in dicots have resulted in the elucidation of mechanisms involved in the transition from vegetative to reproductive growth, the determination of inflorescence, floral meristem, and floral organ identity (Purugganan et al., 1995; Ferrándiz et al., 2000; Hartmann et al., 2000). The flowers of the well-studied dicot species like Arabidopsis and Antirrhinum consist of four concentric whorls with, from outside to inside, the sepals, petals, stamens, and carpels. The determination of the identity of these floral organs has been extensively studied in these two species using homeotic flower mutants (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991), and these studies resulted in the formulation of the so-called ABC model (for review, see Coen and Meyerowitz, 1991). This model proposes that class A genes specify sepals and, together with class B genes, specify petals. Class B and C genes specify stamens, and C alone determines the identity of carpels. The model was later extended, both in Arabidopsis and Petunia, with the class D genes, necessary for ovule development and with class E genes (*SEP* genes), indispensable for the determination of petal, stamen, carpel, and ovule identity (Angenent et al., 1995; Colombo et al., 1995; Pelaz et al., 2000; Favaro et al., 2003; Ferrario et al., 2003; Pinyopich et al., 2003). The majority of the class A, B, C, D, and E homeotic genes belong to the MADS box transcription factor family and they are characterized by a typical modular structure. The MADS box (M), a highly conserved DNA-binding domain, is located at the N terminus. This domain is followed by a less conserved I region (I) and by the moderately conserved K box (K; keratin-like coiled-coil structure),

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both important for dimerization. The C terminus (C) is the most variable part and is involved in ternary complex formation and transcriptional activation (Egea-Cortines et al., 1999).

The ABC model has been shown to be widely applicable in dicot species (Pnueli et al., 1994; Kater et al., 1998; Berbel et al., 2001; Kater et al., 2001; Immink et al., 2003). Furthermore, MADS box genes that are homologous to the dicot ABC genes have also been identified in monocot species (Mena et al., 1995; Ambrose et al., 2000; Jeon et al., 2000a; Schmitz et al., 2000; Fornara et al., 2003; Xiao et al., 2003). Interestingly, the flower structure of these species is very different. The rice flower, for instance, develops in the outermost whorls two leaf-like structures, the lemma and the palea. Instead of the petals, lodicules develop, which are important structures for opening the flower during anthesis. Comparative studies have tried to give a satisfactory interpretation of the floral organs of rice flowers in respect to dicot flowers. The lodicules are mostly thought to represent the petals (Ambrose et al., 2000), the palea is considered to be the equivalent of the dicot sepals (Schmidt and Ambrose, 1998), and the lemma is considered to be a bract (Clifford, 1987). Investigations on the rice ABC genes have helped these comparative studies and also shown that these classes of genes have a similar function in rice flower development. Nevertheless, while B and C class genes in rice have been shown to control the development of the three inner whorls of the rice flower (Chung et al., 1995; Kang et al., 1995, 1998; Moon et al., 1999a; Kyozuka and Shimamoto, 2002; Xiao et al., 2003), as for their Arabidopsis homologs, the A function still remains controversial. *AP1* of Arabidopsis is the best characterized class A gene, and its activity is necessary for the correct development of sepals and petals and for determining the identity of the flower meristems (Irish and Sussex, 1990; Mandel and Yanofsky, 1995). Furthermore *AP1* is involved in the determination of inflorescence architecture together with the closely related *FUL* and *CAL* genes. *AP1*, *FUL*, and *CAL* act redundantly in the control of meristem identity, and only the *ap1 ful cal* triple-mutant plants exhibit a dramatic nonflowering phenotype, in which flower meristems are converted into leafy shoots (Ferrándiz et al., 2000).

In monocot species like barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), *AP1*-like genes have been demonstrated to be induced by vernalization in winter accessions and are probably involved in the floral transition (Trevaskis et al., 2003; Yan et al., 2003). In rice four MADS box genes (*OsMADS20*, *OsMADS14*, *OsMADS15*, and *OsMADS18*) show high sequence homology with *AP1*. Only for *OsMADS14*, functional studies have been reported by Jeon et al. (2000b). They showed that ectopic expression of *OsMADS14* in rice induced the development of shoot- or embryo-like structures from callus, on which subsequently abnormal flowers developed. Based on these results a real ortholog of *AP1* has not yet been identified in rice.

Here we present the functional analysis of the rice MADS box gene *OsMADS18* which belongs to the *AP1/SQUA* group of MADS box transcription factors (Moon et al., 1999b; Masiero et al., 2002; Pelucchi et al., 2002). *OsMADS18* is widely expressed in all plant tissues. Overexpression of *OsMADS18* in rice indicates a role in the acceleration of the differentiation program of the vegetative shoot meristems. The silencing of *OsMADS18* using an RNA interference (RNAi) approach did not result in any visible phenotypic alteration, suggesting that a redundant background is able to compensate for the absence of *OsMADS18*.

The analysis of *OsMADS18* in Arabidopsis revealed that some of the natural partners of AP1 are able to interact with OsMADS18, suggesting that OsMADS18, when overexpressed in Arabidopsis, can subtract AP1 protein partners from active transcription factor complexes. Although OsMADS18 appears not to be able to complement the AP1 function, it seems that the selectivity for protein partners is still conserved between these distantly related monocot and dicot MADS box proteins.

RESULTS

OsMADS18 Is an *AP1/SQUA*-Like MADS Box Gene

OsMADS18 is a rice MADS box transcription factor belonging to the *AP1/SQUA* clade of MADS box genes (Moon et al., 1999b; Pelucchi et al., 2002). An alignment of the amino acid sequences of OsMADS18 with FUL and AP1 of Arabidopsis, ZAP1 of maize, and other rice proteins belonging to the AP1/SQUA clade is presented in Figure 1. Interestingly, all the monocot proteins have a conserved sequence in the terminal part of the protein (LPPWML), which is also present in FUL but not in AP1 (Fig. 1; see also Litt and Irish, 2003; Vandenbussche et al., 2003). Furthermore, they lack the C-terminal amino acid motif (CFAA), present in AP1, which is recognized as the target for prenylation (Fig. 1). This sequence is likely to play an important role in the determination of the function and specificity of AP1 in Arabidopsis (Yalovsky et al., 2000). These data suggest that OsMADS18 and all other rice AP1/SQUA-type proteins are more closely related to FUL than to AP1.

Northern-blot analysis demonstrated that *OsMADS18* transcripts could be detected in roots, leaves, inflorescences, and developing kernels (Masiero et al., 2002); however, expression analysis using seedlings showed that *OsMADS18* is not expressed at early stages of plant development (Fig. 2). The transcripts are first detected in leaves 4 weeks after germination, and expression levels are increased when the plant reaches the reproductive stage (Fig. 2).

We analyzed by in situ expression experiments the spatial and temporal expression pattern in vegetative tissues and inflorescences. These experiments showed that after 30 d from germination the transcripts are

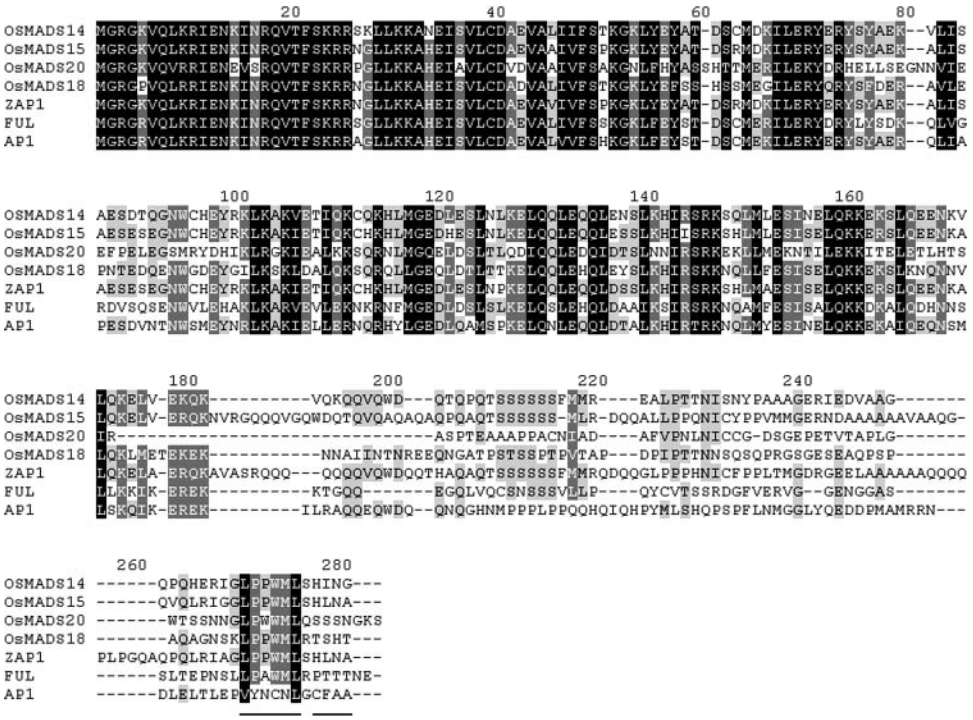

Figure 1. Sequence alignment of AP1 and FUL of Arabidopsis; ZAP1 of maize; and OsMADS18, OsMADS14, OsMADS15, and OsMADS20 of rice. Black boxes indicate fully conserved residues, shaded boxes indicate similar and partially conserved residues. The MADS box region spans amino acids 1 through 58, the I region spans amino acids 61 through 93, and the K box spans amino acids 96 through 161.

present at high levels in the apical meristem and in the meristematic leaf primordia formed on its flanks. The same expression pattern was observed in the axillary vegetative meristems (Fig. 3A). Furthermore *OsMADS18* is strongly expressed in adventitious root primordia formed at the base of the culm (Fig. 3B), but not in the parenchyma across which the adventitious root is elongating.

After the floral transition, as the inflorescence develops, *OsMADS18* transcripts are localized in the developing panicle at the early stage of secondary rachis-branch primordia differentiation. *OsMADS18* is not expressed in the elongating inflorescence branches but is restricted to the meristematic domes that will subsequently give rise both to spikelets and to secondary branches (Fig. 3C). The transcript is also present in the procambium of the rachis branches and in all floral organ primordia (Fig. 3D). These analyses show that *OsMADS18* is expressed in all parts of the plant with high expression levels in the roots and flower meristems.

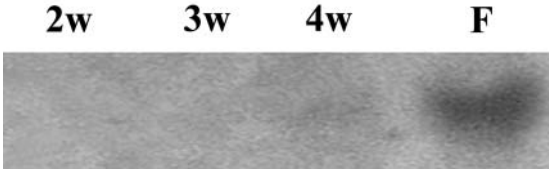

Figure 2. *OsMADS18* expression in leaves. Northern-blot analysis using wild-type leaves at different developmental stages. 2w, Two weeks after germination; 3w, 3 weeks after germination; 4w, 4 weeks after germination; F, flowering time.

OsMADS18 Functional Analysis in Rice by *RNAi* and Overexpression Approaches

RNAi-Mediated Silencing of OsMADS18

We used an *RNAi*-based approach to silence *OsMADS18* in rice. A specific portion of the *OsMADS18* cDNA, lacking the highly conserved MADS box and part of the I region, was cloned in antisense and sense orientation in an RNAi expression cassette, under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The construct was transformed into rice by Agrobacterium-mediated transformation. A total of 31 independent hygromycin-resistant calli were obtained. For each of these calli one regenerated plant was analyzed in detail. The *RNAi* approach proved to be very efficient in silencing *OsMADS18* since 60% of the lines showed reduction of transcript levels to various degrees (Fig. 4). For more than 80% of these lines *OsMADS18* mRNAs could not be detected by northern-blot analysis while the remaining 20% still expressed *OsMADS18*, although very weakly. Both the 31 T_0 plants and the T_1 progeny of 10 selected transformants were normal in development. No visible alterations were observed in panicle

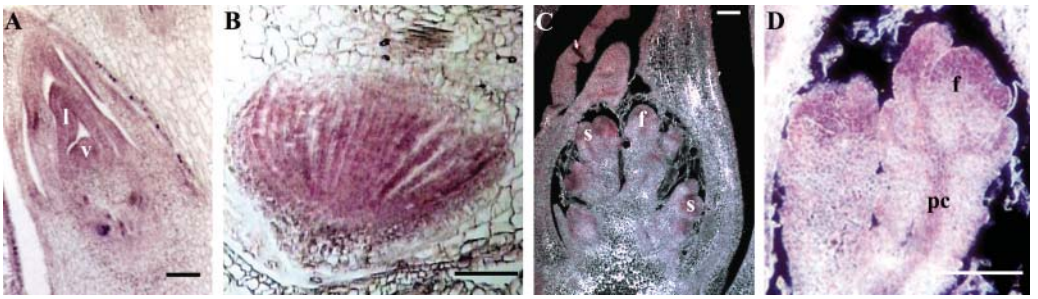

Figure 3. *OsMADS18* expression analysis in vegetative and reproductive tissues by in situ hybridization. A, Axillary bud meristem 30 d after germination with meristematic leaf primordia. B, Adventitious root primordium protruding from the culm cortex 30 d after germination. C, Developing panicle at the early stage of secondary rachis-branch primordia differentiation. D, Close up of a flower primordium 50 d after germination. The hybridization signal is present in the meristematic domes of the flower and in the procambium forming the vascular bundle. v, Vegetative meristem; l, leaf primordium; s, secondary branch primordium; f, flower primordium; pc, procambium. A and B are bright field pictures, C and D are dark field pictures. Bars represent 100 μm in A and C, bars represent 50 μm in B and D.

and flower morphology. Furthermore, we analyzed these plants for differences in flowering time under inductive short day (12 h light/12 h dark) and non-inductive long day (16 h light/8 h dark) conditions. This analysis showed that the flowering time of the RNAi plants is comparable to wild-type plants (data not shown). These observations suggest that other genes are possibly redundant with *OsMADS18*. Possible candidates for such a role, as inferred from phylogenetic analysis, are *OsMADS14*, *OsMADS15*, and/or *OsMADS20* (Lee et al., 2003).

Overexpression of OsMADS18 in Rice

To address the function of *OsMADS18* in rice we constructed an overexpression cassette, fusing the *OsMADS18* coding sequence with the strong CaMV35S promoter. Twenty-seven independent transgenic lines that overexpressed the transgene at different levels were identified (data not shown). Four of these plants that showed the highest levels of *OsMADS18* expression remained very small in size and flowered at 105 d after germination compared to wild-type plants which flower at 140 d after germination (Fig. 5A). Two of them (501S and 1102S) were selected for further studies. Expression analysis of progeny plants of line 501S and 1102S demonstrated that *OsMADS18* overexpression segregated with the early flowering phenotype (data not shown).

In order to test whether *OsMADS18* overexpression affected only the transition to flowering or had a broader effect on rice development, we carried out a detailed morphological analysis on plants, ranging from 0 to 30 d after germination (Fig. 5D). The first effects can already be observed 5 d after germination (Fig. 5, B and C). At this time leaves of transgenic plants are still enclosed by the coleoptile, while wild-type leaves are already emerging from it. After 7 d from germination wild-type plants are about 12 mm long while the transgenic 501S and 1102S plants are 5.5 mm on average (Fig. 5D). Lines 501S and 1102S stay smaller than wild-type plants and this effect is due to a lower rate of internode elongation (Fig. 6, D–G) and a reduction in the length of the leaf sheath. Despite this difference, leaf number is comparable between wild-type and transgenic lines. Regardless of this deficiency in elongation ability, mutant lines form axillary meristems earlier than wild-type plants. These axillary buds are visible in lines overexpressing *OsMADS18* after 7 d from germination (Fig. 6, A and B), whereas in wild-type plants these buds develop only after 15 d

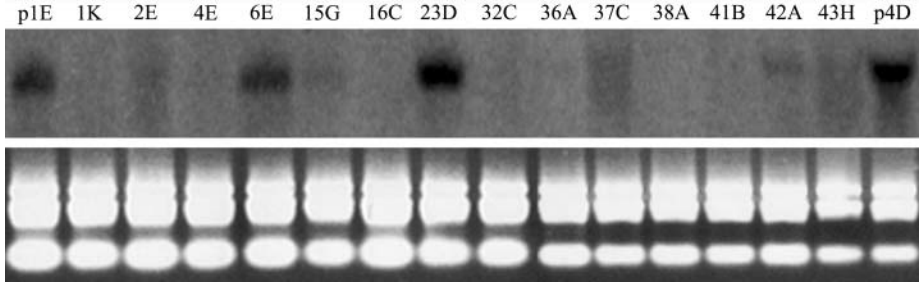

Figure 4. Expression analysis on *OsMADS18* RNAi primary transformants. Total RNA was extracted from leaves of regenerated plants and used for northern-blot analysis. Hybridization was done using a probe specific for OsMADS18. Each lane represents an independent transformant. p1E and p4D are samples taken from two independent plants transformed with the empty vector. RNA quality and equal loading was checked by ethidium bromide staining (lower section).

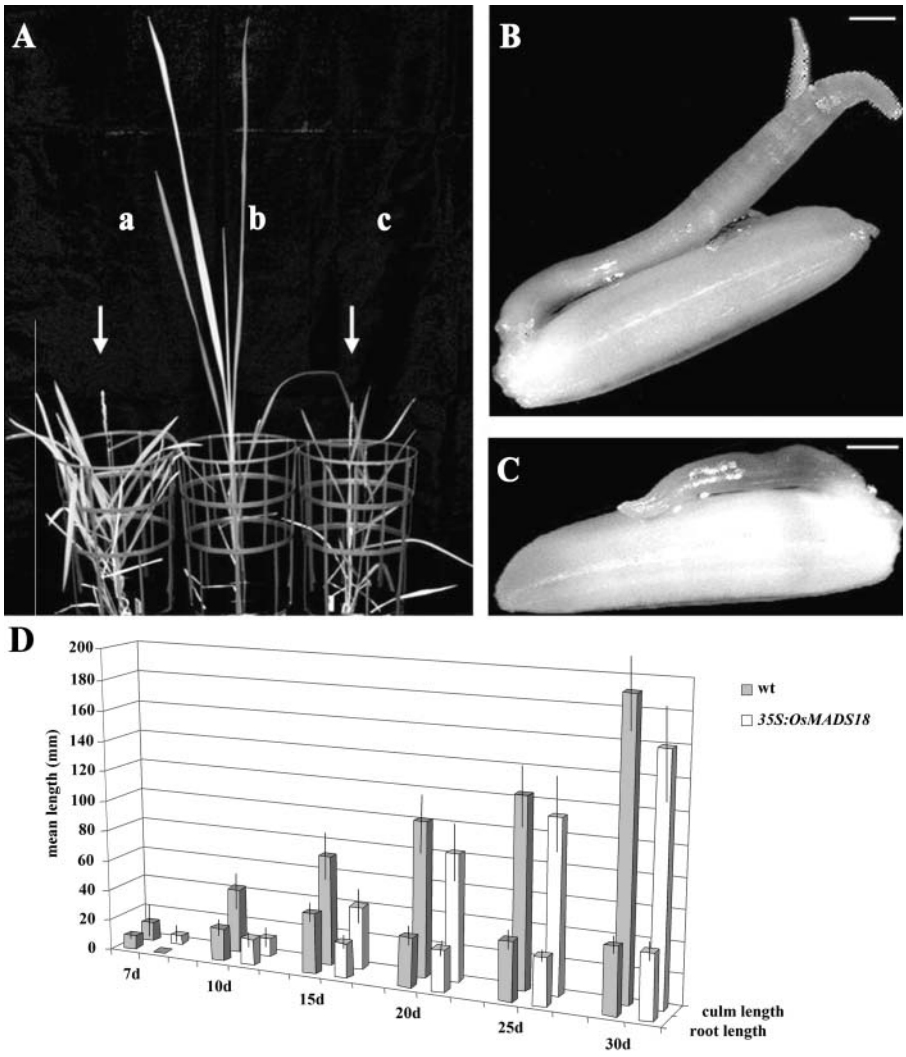

Figure 5. Analysis of *35S:OsMADS18* plants. A, Transgenic plants overexpressing *OsMADS18* (a and c) flower earlier compared to wild type (b). The arrows indicate the emerging inflorescences of the transgenic plants. B and C, Stereomicroscope images of a wild-type (B) and *35S:OsMADS18* seedling (C) 5 d after germination. The leaves of the transgenic plant are enclosed in the coleoptile (C), whereas hypocotyl elongation and leaf expansion have already occurred in the wild type (B). Bars represent 1 mm. D, Mean length of adventitious roots (first row), and mean length of the culm (second row) of wild-type (gray columns) and *35S:OsMADS18* lines (black columns) after 7, 10, 15, 20, 25, and 30 d from germination. Bars indicate the SES of the means.

from germination (Fig. 6C and Supplemental Fig. 1, available at www.plantphysiol.org). Furthermore, in the leaves of the transgenic plants the aerenchyma differentiates earlier than in wild-type plants and the aerenchyma cavities are larger (Fig. 6, A and B).

We also monitored the effects on root development in the transgenic lines 501S and 1102S. Microscopic analysis revealed that the adventitious root primordia develop at the same time as in wild-type plants although their number was reduced in these transgenic lines. Furthermore, at early stages the adventitious root elongation in lines 501S and 1102S is slower compared to wild-type plants (Fig. 5D; Supplemental Table I).

The differences between wild-type and transgenic lines are more evident shortly after germination but, as the plants proceed in development, the developmental gap between wild-type and transgenic lines is progressively reduced (Fig. 5D; Supplemental Table I). After 30 d from germination the number and length of adventitious roots in wild-type and transgenic lines are comparable although in the transgenic lines the aerenchyma is still at a more advanced stage of development (Fig. 6, H and I).

Expression of *OsMADS18* in Arabidopsis Causes an *ap1* Mutant Phenotype

AP1/SQUA-like genes, when overexpressed, generally cause an early flowering phenotype. To investigate whether *OsMADS18* also induces early flowering in Arabidopsis we ectopically expressed *OsMADS18* in this heterologous system. No significant effect on flowering time was observed, however, surprisingly, 10% of the plants (of a total of 100 transformants) showed floral phenotypes that were very similar to the *ap1* mutant (Fig. 7H; Irish and Sussex, 1990; Bowman et al., 1993). The mildest phenotypes show only a reduction in sepal and petal size (Fig. 7B). The result is

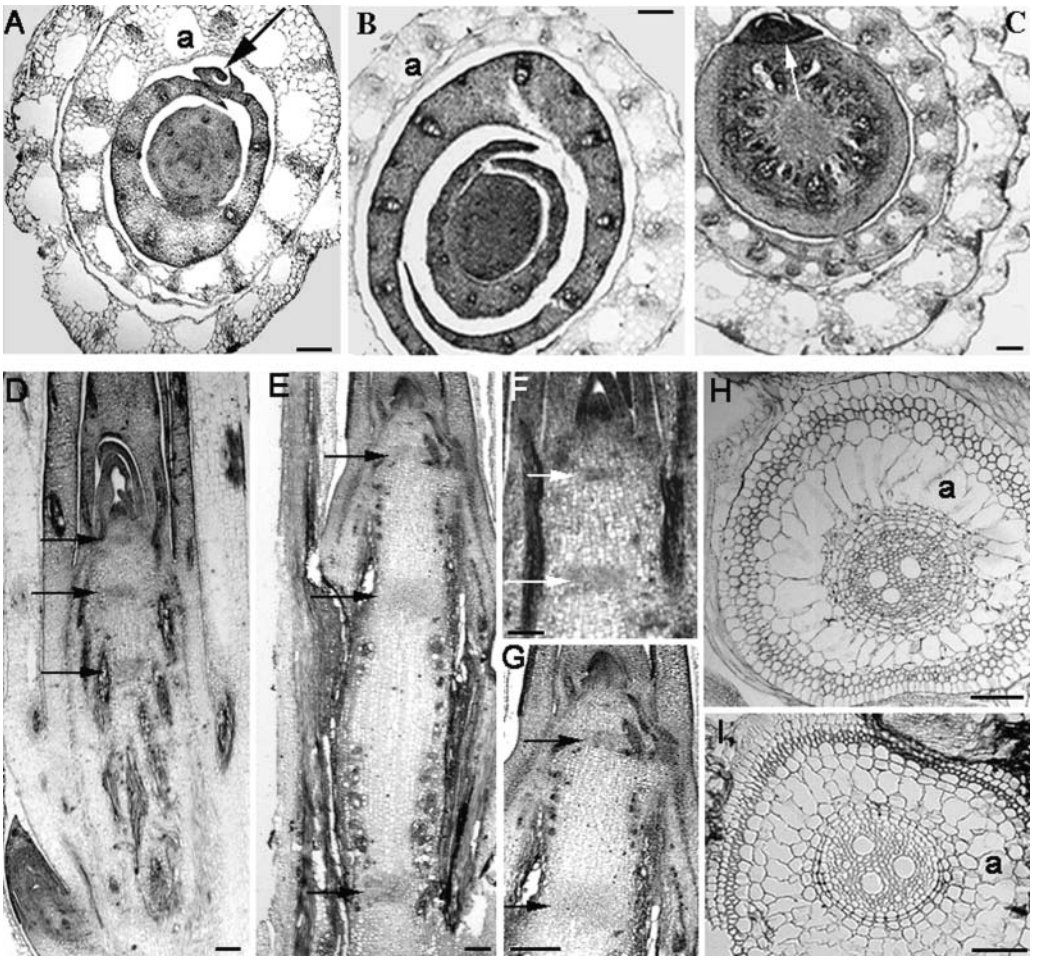

Figure 6. Histological analysis of *35S:OsMADS18* transgenic plants (A, D, F, and H) and of wild-type plants (B, C, E, G, and I) at various days from germination. A to C, H, and I are transverse sections, D to G are longitudinal sections. A and B, Axillary bud (arrow) differentiated in *35S:OSMADS18* lines (A) and not in the wild-type plants (B) after 7 d (sections at the same distance from the shoot apex). The differentiation of aerenchyma (a) is more precocious in transgenic than in wild-type plants. C, The axillary bud (arrow) is present in the wild type after 15 d. D and E (day 30), Internodes are shorter in the *35S:OSMADS18* lines (D) compared to the wild type (E). The arrows show the meristematic regions of the nodes. F and G, Close-up pictures of D and E, showing the shoot region with two apical nodes at higher magnification. H and I, Comparison between the adventitious roots of transgenic plants (H) and wild type (I). Root cortex aerenchyma (a) is more developed in transgenic plants. (Bars represent 100 μm in A–E and G; bars represent 50 μm in F and H–I).

that the pistil is not enclosed by the perianth organs and protrudes from the flower. Plants having an intermediate phenotype have flowers that in the first whorl develop leaf-like organs bearing stellate trichomes, which is typical for cauline leaves (Fig. 7C), while wild-type sepals have simple trichomes (Fig. 7A).

Around 5% of the plants showed more severe phenotypes. Some of the first-whorl organs were homeotically converted to carpelloid organs on which ovules developed (Fig. 7F). In these severely affected flowers the petals were, in general, completely absent (Fig. 7, E and F). Frequently the most affected plants had flowers from which extra flowers arose from the axils of the first whorl organs (Fig. 7) and this pattern was reiterated producing tertiary and even quaternary flowers (Fig. 7G).

In *CaMV35S:OsMADS18* Arabidopsis Plants *AP1* Expression Is Not Affected

One of the possible explanations for the *ap1* phenotypes that we observed in the Arabidopsis plants that expressed *OsMADS18* could be that in these transgenic plants the expression of the endogenous *AP1* gene is repressed. To verify this possibility we performed a reverse transcription (RT)-PCR analysis to check for the expression of *AP1* in these transgenic plants. Figure 8 shows the RT-PCR products obtained using RNA extracted from transgenic and control

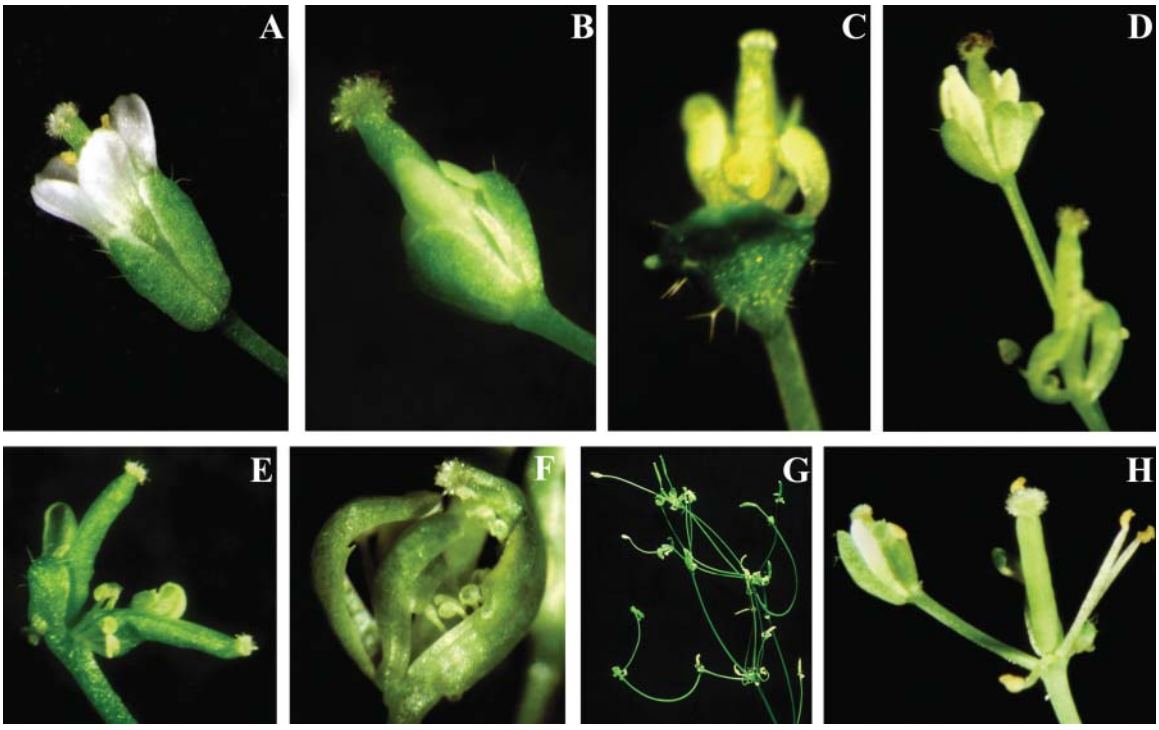

Figure 7. *OsMADS18* overexpression in Arabidopsis. A, Wild-type flower. B, Weakly affected flower showing reduction in the size of petals and sepals. C, Weakly affected flower in which normal petals develop and sepals are converted into leaf-like structures that differentiate stellate trichomes (arrow). D and E, Strongly affected flowers that develop a new flower at the axil of a first whorl organ. F, Severe flower phenotype in which first whorl organs develop carpelloid characteristics. Stigmatic papillae are evident at the tip of the organs and ovules develop along their margins. G, Tertiary and quaternary flowers arise at the axil of the first whorl organs in most affected flowers. H, An *ap1-10* mutant flower.

wild-type flowers. These analyses show that *AP1* expression is not affected in these transgenic plants.

OsMADS18 Interacts With MADS Box Factors That Form Dimers With AP1

Another possible explanation for the observed *ap1* phenotype in the *OsMADS18*-expressing Arabidopsis plants is that the OsMADS18 protein interacts with proteins that normally dimerize with AP1, since OsMADS18 shows significant sequence homology over a large part of the protein (Fig. 1). When these OsMADS18-containing protein complexes are not fully functional, this interaction could result in a dominant negative *ap1* mutant phenotype.

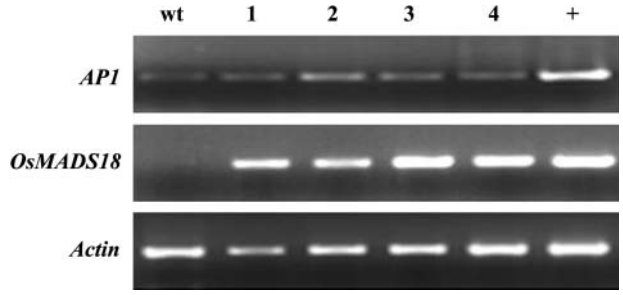

Figure 8. RT-PCR on leaves of wild-type and transgenic *35S:OsMADS18* plants. 1, *OsMADS18* overexpressing line showing no visible phenotype. 2 to 4, *OsMADS18* overexpressing lines showing flower phenotypes described in Figure 7D, F, and G, respectively. +, positive control.

Yeast Two-Hybrid Assay Using OsMADS18 as a Bait

Recently we used *OsMADS18* for a yeast two-hybrid screen to identify proteins that interact with this MADS box factor (Masiero et al., 2002). The complete *OsMADS18* open reading frame, with the exception of the Met initiation codon, was cloned in the vector GAL4-BD in frame with the DNA-binding domain of GAL4. This construct was used to screen a rice cDNA expression library made from RNA extracted from leaf, inflorescence, and developing kernel tissues (see Masiero et al., 2002). This yeast two-hybrid screen resulted in the identification of the MADS box factors OsMADS24, OsMADS45, OsMADS6, and OsMADS47. The interaction between OsMADS18 and OsMADS6 has been reported previously by Moon et al. (1999b), which used OsMADS6 as a bait. Apart from the MADS box encoding cDNAs, an additional non-MADS box

clone was isolated encoding a CCAAT-binding protein complex subunit, NF-YB (Masiero et al., 2002). OsMADS24 (also called OsMADS8) and OsMADS45 (also called OsMADS7) are the rice MADS box factors that show high sequence identity with the Arabidopsis SEP proteins. Whereas OsMADS6 shows the highest homology with AGL6, and OsMADS47 is similar to SVP and AGL24 from Arabidopsis (Ma et al., 1991; Greco et al., 1997; Moon et al., 1999b; Hartmann et al., 2000; Pelaz et al., 2000; Yu et al., 2002; Michaels et al., 2003). All the MADS box clones isolated by the two-hybrid screen were partial cDNAs. *OsMADS24* and *OsMADS45* lacked sequences corresponding to 8 and 10 amino acids, respectively, at the N terminus of the MADS domain. The longest *OsMADS6* clone that we isolated started at nucleotide 204, lacking the whole MADS domain and part of the I region. The longest clone obtained for *OsMADS47* lacked the whole MADS domain and the I region. These observations indicate that, at least for these MADS box proteins, the K region is probably sufficient to establish the interaction.

To confirm the interaction between OsMADS18 and its putative partners, the complete open reading frames of *OsMADS47*, *OsMADS24*, *OsMADS45*, and *OsMADS6* were cloned in frame with the activation domain of GAL4 in the vector GAL4-AD. The plasmids obtained were named 47-AD, 24-AD, 45-AD, and 6-AD. Yeast strain PJ694A (James et al., 1996) was cotransformed with the binding domain vector containing OsMADS18 (18-BD) and one of each of the activation domain vectors (47-AD, 24-AD, 45-AD, and 6-AD). Yeast strain PJ694A allows selection for three different reporter genes: adenine, His, and LacZ. The yeast transformants obtained were all able to activate each of the reporter genes. When selected for activation of the *HIS3* reporter, different concentrations of 3AT were added to the media to examine the stability of the interaction. Each of the OsMADS18 partners was able to grow on medium containing high concentrations of 3AT (5 mM), indicating that all the interactions were stable (Table I).

Table I. *OsMADS18 interactions tested with yeast two-hybrid assays*

BD-OsMADS18	Selective Media			
	-W-L-H	-W-L-$H_{3AT\ (2Mm)}$	-W-L-$H_{3AT\ (5Mm)}$	-W-L-A
AD-OsMADS24	++[a]	++	++	++
AD-OsMADS45	++	++	++	++
AD-OsMADS6	+++	+++	+++	+++
AD-OsMADS47	++	++	++	++
AD-OsMADS18	−	−	−	−
AD-SEP1	++	++	++	++
AD-SEP2	−	−	−	−
AD-SEP3	++	++	++	++

[a]The + symbols indicate the relative strength of the interaction as determined by the growth of the yeast colonies on selective media; −, no growth.

To investigate whether OsMADS18 was able to form homodimers the *OsMADS18* open reading frame was also cloned as an activation domain fusion protein. Both the 18-BD and 18-AD vectors were introduced into PJ694A and their interaction was assayed by growth on selective media and by β-galactosidase assays. The lack of activation of the markers showed that OsMADS18 is not able to form homodimers (Table I).

In Vitro Confirmation of Two-Hybrid Interactions

In order to confirm the observed yeast two-hybrid interactions with OsMADS18, coimmunoprecipitation experiments between OsMADS18 and its partner OsMADS24 were performed (Fig. 9). OsMADS24 was produced by in vitro transcription-translation, including ^{35}S-Met, whereas OsMADS18 was expressed in *Escherichia coli* as a thioredoxin (TRX) fusion protein. The protein extracts containing the ^{35}S-labeled OsMADS24 and the TRX-OsMADS18 fusion protein were mixed with G protein and antibodies raised against the thioredoxin domain. A control incubation was performed using a mixture lacking OsMADS18. The protein G-agarose was washed several times and proteins were subsequently separated by SDS-polyacrylamide gel-electrophoresis. Labeled OsMADS24 was detected by autoradiography only when OsMADS18 was present in the protein mixture, indicating that OsMADS24 specifically interacts with OsMADS18.

OsMADS18 Interacts with SEP Proteins from Arabidopsis

As reported by Pelaz et al. (2001) proteins interacting with AP1 are SEP3, SVP, AGL24, and SOC1. SEP3 is the strongest interactor and is necessary for normal petal development (Honma and Goto, 2001; Pelaz et al., 2001). To confirm that OsMADS18 can indeed interact with protein partners of AP1 we tested by two-hybrid assays whether OsMADS18 interacts with SEP3. These assays showed that OsMADS18 indeed forms dimers with SEP3 of Arabidopsis (Table I). Furthermore, we tested also SEP1 and SEP2, which are highly homologous to SEP3. In these assays only SEP1 shows a clear interaction with OsMADS18. When we checked AP1 for interactions with the SEP proteins we obtained the same result: AP1 interacts only with SEP1 and SEP3 and not with SEP2 (Table II). It could well be that the interaction with SEP2 is somehow less stable in yeast, which might be different in plant cells.

To investigate further the evolutionary conservation of the AP1 and OsMADS18 interactions we also tested AP1 for interaction with the rice homologs of SEP3 (Table II). These assays showed that AP1 makes stable interactions with OsMADS24 and OsMADS47. These results indicate that AP1 and OsMADS18 each form heterodimers that are conserved between monocot and dicot plants.

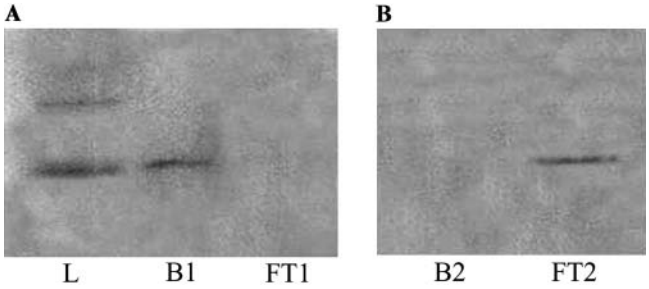

Figure 9. In vitro binding of OsMADS18 and OsMADS24. OsMADS18 was produced in *E. coli* as a TRX fusion protein, and *OsMADS24* was translated in vitro in the presence of ^{35}S-Met. OsMADS18 and OsMADS24 were mixed and loaded on a G protein anti-TRX antibody column. The flow-through was collected and the column was washed several times. A control experiment was performed without adding OsMADS18. The protein G-protein complex was separated by SDS-PAGE and ^{35}S-Met labeled OsMADS24 was detected by autoradiography. A, L, ^{35}S-Met labeled OsMADS24 in vitro translation product. B1, Protein G protein complex. FT1, Flow-through fraction. B, Control column. B2, Protein G protein complex. FT2, Flow-through fraction.

DISCUSSION

OsMADS18 Belongs to the FUL Lineage of MADS Box Transcription Factors

In our effort to isolate and study MADS box genes involved in flower development and floral transition, we started with the functional characterization of *OsMADS18*. Phylogenetic reconstruction placed OsMADS18 in the AP1/SQUA clade, in close relationship to AP1 and FUL from Arabidopsis and SQUA from Antirrhinum. Recently, Litt and Irish (2003) showed that the monocot lineage evolved only FUL-like proteins, while AP1-like proteins seem to be characteristic of the eudicot (eu) clade. Differences in the C-terminal domain are responsible for this splitting since FUL-like sequences present a highly conserved hydrophobic box (L/MPPWML), which cannot be found in the euAP1 clade. The euAP1 lineage instead shows two typical conserved motifs, RRNaLaLT/NLa and a farnesylation domain (CFAT/A) that terminates the protein (Litt and Irish, 2003; Vandenbussche et al., 2003). OsMADS18 shows the typical FUL-like LPPWML motif and lacks the ending farnesylation box supporting for a derivation from the FUL-lineage. Farnesylation was shown to be important for the correct function of AP1 both in vivo and in vitro, but it is interesting to note that the absence of a farnesylation domain in FUL-like proteins from both monocots and dicots doesn't seem to alter their ability to complement the *AP1* function in an *ap1* mutant background (Yalovsky et al., 2000; Berbel et al., 2001; Gocal et al., 2001; Jang et al., 2002).

OsMADS18 is widely expressed in rice, as revealed by expression analysis in flowering plants (Pelucchi et al., 2002). Detailed expression analysis during early stages of plant development showed that the *OsMADS18* transcripts are present in all tissues analyzed and accumulate to higher levels in organs that retain meristematic characteristics like the adventitious root meristems, primary and axillary shoot meristems, secondary inflorescence branches meristems and in the procambial strand region. The expression in the flower meristem and in the floral organ primordia is common for members of the *AP1/SQUA* subfamily of MADS box genes (Huijser et al., 1992; Mandel et al., 1992; Mena et al., 1995; Moon et al., 1999b; Kyozuka et al., 2000). Furthermore, the expression of *OsMADS18* in the roots and procambium is similar to that reported for *LtMADS1* of *Lolium temulentum* and of *SaMADSB* of *Sinapsis alba* (Menzel et al., 1996; Gocal et al., 2001) and suggests a role of this gene in the specific development of these tissues.

OsMADS18 transcripts can be detected in rice leaves only after 4 weeks from germination, and the increase in steady state mRNA levels correlates with the progressive acquisition of flowering competence. Also in other monocot species, like barley and wheat, the switch to the reproductive stage is marked by an increase in expression of *AP1/SQUA*-like genes. In winter accessions of wheat, *Triticum monococcum*, and barley, the *WAP1*, *TmAP1*, and *BM5* genes, respectively, show a marked increase in transcript levels during the induction of the reproductive phase (Trevaskis et al., 2003; Yan et al., 2003). In spring accessions of wheat and barley that do not require a cold treatment to flower, the expression of these genes is independent of a cold treatment. Since rice is a subtropical species it does not require a vernalization treatment. Nevertheless, also in rice we observed increased levels in *OsMADS18* expression when plants are in the reproductive phase. These observations confirm the idea that in monocot species increased levels of *AP1/SQUA*-like gene transcripts are important for the floral transition (Jeon et al., 2000b; Trevaskis et al., 2003; Yan et al., 2003). Despite this general behavior of this class of genes during the floral transition, the pathways that lead to the change in their expression are probably different, since in vernalization-dependent species their expression is regulated by cold, whereas in subtropical species like rice other pathways are probably regulating the *AP1/SQUA*-like genes.

Table II. *AP1 interaction tested by yeast two-hybrid*

BD-AP1	Selective Media			
	-W-L-H	-W-L-H$_{3AT\ (2Mm)}$	-W-L-H$_{3AT\ (5Mm)}$	-W-L-A
AD-SEP1	+++	+++	+++	+++
AD-SEP2	−	−	−	−
AD-SEP3	+++	+++	+++	+++
AD-OsMADS24	+++	+++	+++	+++
AD-OsMADS45	++	++	++	++
AD-OsMADS6	−	−	−	−
AD-OsMAD18	−	−	−	−

Functional Analysis of *OsMADS18* Reveals a Role in the Promotion of Flowering in Rice

Since increased expression of *AP1/SQUA*-like genes in monocots is correlated with the floral transition we investigated whether alterations in *OsMADS18* expression caused changes in flowering time. Transgenic rice plants overexpressing *OsMADS18* showed an early flowering phenotype with a heading date that was anticipated 5 weeks when compared to the wild type. All rice MADS box genes belonging to the *AP1/SQUA* subfamily characterized so far, have been shown to cause early flowering when overexpressed (Chung et al., 1994; Kang et al., 1997; Jeon et al., 2000b). This effect is similar to that shown by genes such as *AP1* and *FUL* when ectopically expressed in Arabidopsis (Mandel and Yanofsky, 1995; Ferrándiz et al., 2000). Thus *OsMADS18* retains the ability, as other *AP1/SQUA*-like genes, to induce flowering although it is not clear from these experiments whether flowering is promoted directly by *OsMADS18* or through repression of a negative regulator.

Ectopic expression of *OsMADS18* induced precocious initiation of axillary shoot meristems. This observation together with the early transition to flowering suggests that *OsMADS18* is able to promote the differentiation program of the vegetative shoots. This effect is not maintained for the entire life cycle but is progressively reduced, and when the plants are at 30 d after germination there is almost no difference anymore between the wild-type and transgenic plants. Assuming that *OsMADS18* is able to stimulate shoot differentiation, its ectopic expression at early stages of plant development (when *OsMADS18* normally is not expressed) might cause the observed acceleration. Whereas at later stages when *OsMADS18* is normally expressed, the overexpression of this gene has no significant effect.

Silencing of *OsMADS18* through an RNAi approach did not result in any phenotypic effect when compared to wild-type plants. Although RNAi-based gene silencing is very effective in rice, as already reported by Hayama et al. (2003) and Xiao et al. (2003), the redundancy with other members of the *AP1/SQUA* subfamily may have masked the role of *OsMADS18*. Possible candidates for redundancy are *OsMADS14*, *OsMADS15*, and/or *OsMADS20* (Moon et al., 1999b; Kyozuka et al., 2000; Jang et al., 2002; Lee et al., 2003).

Overexpression of *OsMADS18* in Arabidopsis Causes an *ap1* Mutant Phenotype

By expression of *OsMADS18* in Arabidopsis we investigated whether this rice gene can stimulate flowering, as has been shown for the *SQUA*-like genes *AP1* and *FUL* (Mandel and Yanofsky, 1995; Ferrándiz et al., 2000). In Arabidopsis plants overexpressing *AP1* the inflorescence shoot meristems are converted into flower meristems and the plants flower dramatically early compared to wild type (Mandel and Yanofsky, 1995). When *OsMADS18* was expressed in Arabidopsis, flowering occurred at the same time as wild type but, strikingly, 10% of the plants showed a flower phenotype closely resembling that of *ap1* (Irish and Sussex, 1990; Bowman et al., 1993).

Although the severity of the observed phenotypes varied between the different transgenic lines, their phenotypes were always similar to those previously described for *ap1* mutants (Bowman et al., 1993; Schultz and Haughn, 1993).

The *ap1* mutant phenotype observed in plants expressing *OsMADS18* is not due to silencing of *AP1*, since *AP1* is normally expressed in these transgenic plants. Since AP1 forms ternary complexes to exploit its functions, OsMADS18 might interact with the same partners as AP1 giving rise to inactive complexes and acting as a dominant negative factor. It has been reported that AP1 interacts with SEP proteins, SVP and AGL24 (Pelaz et al., 2001). Interestingly, OsMADS18 shares the same kind of protein partners in rice as AP1 has in Arabidopsis. OsMADS18 can interact in yeast with OsMADS24 and OsMADS45 (SEP-like), OsMADS47 (SVP/AGL24-like), and OsMADS6 (AGL6-like, although an interaction between AGL6 and AP1 has never been reported).

The two-hybrid screen for OsMADS18 partners in rice resulted in isolation of partial proteins although none of the sequences lacked the regions corresponding to the K box. We dissected the OsMADS18 domains to identify those important for dimerization and confirmed that only those containing a K box were able to interact with OsMADS18 partners (Supplemental Table II). This confirms previous reports that the K box is important for MADS box protein dimerization (Krizek and Meyerowitz, 1996; Fan et al., 1997; Moon et al., 1999b; Lim et al., 2000; Pelaz et al., 2001; Yang et al., 2003). The fact that OsMADS18 interacts with the same type of protein partners as AP1, together with the observation that the K box is highly conserved between AP1 and OsMADS18, strongly suggested that OsMADS18 also interacts with partners of AP1. To verify this possibility we tested the interaction between the SEP proteins and OsMADS18. These experiments demonstrated that OsMADS18 strongly interacts with SEP1 and SEP3 while no interaction was found between OsMADS18 and SEP2. Interestingly in our experiments also AP1 could not interact with SEP2.

It is very likely that the observed *ap1* phenotype is caused by the formation of complexes between OsMADS18 and AP1 partners, which might be able to bind to the same target genes as those containing AP1. However, those containing OsMADS18 are probably functionally inactive, blocking the binding of the functional AP1 complexes. In this scenario the dominant negative effect is not due to the subtraction of SEP proteins from active complexes but more likely involves competition between active and nonactive complexes on the promoters of *AP1* target genes.

The dominant negative effect is not observed in rice flowers when we overexpressed *OsMADS18* since the

complexes formed with OsMADS18 are functional, activating the natural set of target genes. Furthermore, the fact that we do not observe any floral phenotypic effect indicates that the increased *OsMADS18* mRNA levels do not interfere with normal flower development.

As OsMADS18 is able to interact with at least a subset of the AP1 partners in yeast we demonstrated that AP1 can, vice versa, interact with OsMADS24 and OsMADS45 but not with OsMADS6. It's clear from these experiments that, although different in expression pattern, OsMADS18 and AP1 still retain the ability to interact with phylogenetically related partners. The analysis of conserved interactions can be used to compare putative functional homologs as already demonstrated for other rice MADS box factors (Moon et al., 1999a; Favaro et al., 2002; Immink and Angenent, 2002).

It seems likely that a parallel exists between complexes formed in rice and Arabidopsis and, although their functionality cannot be fully restored when corresponding partners are exchanged, they strongly indicate their evolutionary common origin.

MATERIALS AND METHODS

Plant Material

For the overexpression and RNAi-mediated silencing of *OsMADS18*, the *indica* cultivar *Cypressus* and the *japonica* cultivar *Nipponbare* were used, respectively. Overexpression of *OsMADS18* in Arabidopsis was carried out in the *Wassilewskjia* ecotype.

Production and Growth of Transgenic Plants

Transformation of rice (*Oryza sativa*) was carried out by cocultivation of *Agrobacterium tumefaciens* and embryogenic scutellum-derived calli from mature rice seeds as described by K. D'Halluin and E. Gobel (1992; Publication of the International Application under the Patent Cooperation Treaty WO 92/09696) and by Hiei et al. (1994). Regenerated rice plants were grown in greenhouse with a light/dark cycle of 12 h/12 h. Supplementary artificial light was provided. The temperature was artificially maintained at 28°C during the day and at 22°C by night.

Arabidopsis was transformed according to the floral dip method as described by Clough and Bent (1998). The plants were grown at 22°C under long-day conditions (16 h light/8 h dark).

Sequence Analysis

Protein sequences were aligned using the multiple alignment mode of the ClustalX software (ver. 1.83), visualized and edited with the GENEDOC utilities (ver. 2.3.000; www.psc.edu/biomed/genedoc).

Plasmids Construction

OsMASD18 cDNA was amplified with primers OL13 and OL14 (OL13, GAATTCGGGAGAGGGCCGGTGC; OL14, GTCGACTCATGTGTGACTTGTCCGGAG) to introduce the cloning sites *Eco*RI and *Sal*I. Amplification was followed by digestion, and the DNA fragment was cloned in pBDGAL4 (18-BD). The bait includes the complete coding sequence of *OsMADS18*, excluding the initial Met. The bait deletions of *OsMADS18* were also generated by PCR, subsequently digested and cloned in pBDGAL4 as described by Masiero et al. (2002).

The complete *OsMADS18*-coding region was cloned in pET32a to produce the recombinant protein (TRX-OsMADS18), using oligos OL13 and OL14. *OsMADS18* was fused in frame with thioredoxin at the N-terminal domain and with a tail of 6 His residues. This plasmid was used to transform *Escherichia coli* strain *BL21* (*DE3*) *plysS*. The overexpression system based on the T7 promoter was used to produce the recombinant protein (Studier et al., 1990). The TRX-OsMADS18 recombinant protein was purified using an affinity Ni-column (Qiagen, Valencia, CA).

To construct the *35S:OsMADS18* overexpression plasmid, *Bst*XI and *Nhe*I cloning sites were introduced in the *OsMADS18* cDNA by means of PCR. The entire coding region of *OsMADS18* was fused as a *Bst*XI-*Nhe*I fragment in the sense orientation to the 61-bp fragment of the leader sequence from the chlorophyll a/b binding protein gene (cab22L) from petunia (Harpster et al., 1988), to the 535-bp fragment of the CaMV35S promoter, and the 265-bp CaMV 35S terminator sequence fragment at the 3′ end. The *35S:OsMADS18* chimeric gene was introduced into the plant transformation vector pTCV0254 derived from pGSV1 (Cornelissen and Vandewiele, 1989). PTCV0254 contains a 567-bp fragment of the bialophos resistance gene (*BAR*) of *Streptomyces hydroscopicus* under the control of the CaMV 35S promoter (Denecke et al., 1989) between the left and right T-DNA borders.

To construct the *OsMADS18* RNAi plasmid a specific portion of *OsMADS18* (corresponding to nucleotide position 83–280) was amplified with OL668 (GGGGACAAGTTTGTACAAAAAAGCAGGCTTACTGGAGCCAAATACTGAG) and OL669 (GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGTCACACATGAAGGCATCTG). The product of the amplification was cloned into the binary vector pFRH using the Gateway Cloning Technology (Invitrogen, Carlsbad, CA).

Northern-Blot Analysis and RT-PCR

Total RNA was extracted from rice tissues as described by Verwoerd et al. (1989). For northern-blot analysis 10 μg of glyoxal-denatured (1.5 M) total RNA was electrophorized and blotted onto Hybond N^+ membranes (Amersham, Buckinghamshire, UK). A 3′ gene-specific fragment of *OsMADS18* was used for hybridization. Probes were labeled using the Random Priming DNA labeling kit supplied by Roche (Basel). Blots were hybridized as described by Colombo et al. (1998). For RT-PCR analysis, cDNA was produced according to the SuperScript First-Strand Synthesis System (Invitrogen).

Morphological Analysis

For the histological analysis, wild-type seeds and *35S:OsMADS18* seeds were sterilized according to Hiei et al. (1994) and plated on Murashige and Skoog medium, supplemented with 1% (w/v) Suc and 0.8% (w/v) agar (pH 5.8). The seeds were cultured for 40 d at 28°C under 12-h light/d. The germinated seedlings were sampled at 7, 10, 15, 20, 25, and 30 d after germination, fixed in 70% ethanol, dehydrated through a graded ethanol series, and subsequently embedded in paraffin and sectioned at 8 μm. The sections were stained with 0.05% toluidine blue or with eosin and Carazzi's haemalum. A DAS Leica DMRB microscope and a Leica MZ8 stereomicroscope were used for the analyses (Wetzler, Germany). Differences between the mean number of roots and mean length of roots and stems were evaluated at least three times with similar results.

In Situ Hybridization

Developing inflorescences of wild-type plants (*O. sativa* L. cv Nipponbare) were fixed and embedded in paraffin as described by Lopez-Dee et al. (1999). Digoxygenin-labeled (DIG) gene-specific antisense RNA probes were generated by in vitro transcription following the instructions of the in vitro transcription kit (Roche). Hybridization and immunological detection were performed as described by Lopez-Dee et al. (1999).

Yeast Two-Hybrid Screening

Yeast strain HF7c, used for the library screening, was supplied by CLONTECH Laboratories (Palo Alto, CA; Feilotter et al., 1994). The two-hybrid screen was carried out as previously reported by Davies et al. (1996). For the library transformation 60 μg of DNA was used. The interactions between OSMADS18 and its partners were confirmed in yeast strain PJ69-4A (James et al., 1996). PJ69-4A was also used to test the bait deletions of OsMADS18 against 24-AD, 45-AD, 47-AD, and 6-AD. The lacZ test was performed according to Davies et al. (1996).

Coimmunoprecipitation

The TNT-coupled Reticulocyte Lysate system (Promega, Madison, WI) was used to make in vitro translated proteins, and ^{35}S-Met was incorporated to label the protein. To perform coimmunoprecipitation experiments protein G-agarose was used since anti-thioredoxin antibodies have high affinity for it. The conditions employed for coimmunoprecipitation were reported previously by Goto and Meyerowitz (1994).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

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