



Identification of class B and class C floral organ identity genes from rice plants

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Abstract

The functions of two rice MADS-box genes were studied by the loss-of-function approach. The first gene, *OsMADS4*, shows a significant homology to members in the *PISTILLATA (PI)* family, which is required to specify petal and stamen identity. The second gene, *OsMADS3*, is highly homologous to the members in the *AGAMOUS (AG)* family that is essential for the normal development of the internal two whorls, the stamen and carpel, of the flower. These two rice MADS box cDNA clones were connected to the maize ubiquitin promoter in an antisense orientation and the fusion molecules were introduced to rice plants by the *Agrobacterium*-mediated transformation method. Transgenic plants expressing antisense *OsMADS4* displayed alterations of the second and third whorls. The second-whorl lodicules, which are equivalent to the petals of dicot plants in grasses, were altered into palea/lemma-like organs, and the third whorl stamens were changed to carpel-like organs. Loss-of-function analysis of *OsMADS3* showed alterations in the third and fourth whorls. In the third whorl, the filaments of the transgenic plants were changed into thick and fleshy bodies, similar to lodicules. Rather than making a carpel, the fourth whorl produced several abnormal flowers. These phenotypes are similar to those of the *agamous* and *plena* mutants in *Arabidopsis* and *Antirrhinum*, respectively. These results suggest that *OsMADS4* belongs to the class B gene family and *OsMADS3* belongs to the class C gene family of floral organ identity determination.

Introduction

Recent studies on floral homeotic genes from dicots including *Antirrhinum majus*, *Arabidopsis thaliana*, petunia, tomato and tobacco have elucidated that genetic and molecular mechanisms controlling flower organ identity have been highly preserved during angiosperm evolution [9, 39]. Three classes of homeotic genes (A, B, and C) that encode proteins with a very conserved MADS box motif regulate floral organ pattern formation in single or two adjacent whorls among the four whorls of dicot flowers. The class A genes in whorl 1 and C genes in whorl 4 function to develop sepals and carpels, respectively. In combination, the class A and B genes determine the fate of petals in whorl 2. Similarly, the class B and C genes together control formation of stamens in whorl 3. The class A MADS box genes include *APETALA1 (AP1)*

in *Arabidopsis* [12, 25, 39] and *SQUAMOSA (SQUA)* in *Antirrhinum* [15, 39], the class B genes include *APETALA3 (AP3)* and *PISTILLATA (PI)* in *Arabidopsis* [11, 16, 17, 23, 39, 40] and *DEFICIENS (DEF)* and *GLOBOSA (GLO)* in *Antirrhinum* [35, 36, 39], and class C genes include *AGAMOUS (AG)* in *Arabidopsis* [28, 29, 39] and *PLENA (PLE)* in *Antirrhinum* [4, 39]. In addition to specification of stamen and carpel identity, the *AG* and *PLE* genes prevent the indeterminate growth of the floral meristem [29, 4].

Transgenic approaches were undertaken to study the function of the class B MADS box genes in several dicot plants including *Arabidopsis*. Flowers of the transgenic lines constitutively expressing *AP3* under the control of the 35S promoter exhibited a replacement of carpels by stamens, while the sepals remain unaffected [16]. Ectopic expression of both *AP3* and *PI* resulted in the transformation of carpels into sta-

mens and sepals into petals [23]. Ectopic expression of the B function gene *DEF* of *Antirrhinum* in tobacco plants had no effect on floral organ identity, but ectopic expression of the other B function gene, *GLO*, caused homeotic changes in organ identity, producing petaloid sepals and stamenoid carpels [10]. Simultaneous expression of both *DEF* and *GLO* caused more extreme alterations of the floral organs. The conversion of the first whorl sepals to petals was almost complete, accompanying pigment development and morphological alteration [10].

Loss-of-function approaches were also taken to understand the role of the class B MADS box genes. The co-suppression of *FBP1*, the *PI* family in petunia, results in homeotic reversions of petals toward sepals and stamens toward carpels, which resemble the effects of the *Arabidopsis* B function genes [3]. The co-suppression of *pMADS1*, a MADS box gene of the *AP3* family in petunia, resulted in the conversion of petals into sepals. However, there was no change in stamen development, indicating that *pMADS1* is different from the B function genes [38].

Evidence that A and C class genes act antagonistically has been obtained by ectopic expression of *AG* or *AG* homologous genes under the control of the 35S promoter. Transgenic plants expressing the *AG* genes of *Arabidopsis*, *Brassica napus*, petunia, tobacco, tomato, and cucumber showed homeotic conversion of sepals to carpels and petals to stamens, mirroring the *apetala2* (*ap2*) A function mutant phenotype [22, 24, 29, 30, 37, 21]. Although they differ in their ability to determine reproductive organ, it was reported that at least two *AG* homologues exist in petunia and cucumber [21]. Flowers of transgenic plants expressing antisense *AG* genes of *Arabidopsis*, tomato and tobacco phenocopied the *ag* mutant, with floral organ conversion of stamens toward petals and floral meristem indeterminacy in the fourth whorl [22, 28, 31].

In contrast to the ample amount of information on the functional analyses of the MADS box genes in dicots, a limited number of cereal MADS box genes has been studied. The *ZAP1* gene, an *API* homologue in maize, was isolated but its function has not been well characterized [27]. A transposon-induced mutation in *ZAG1*, the maize *AG* homologue [34], did not greatly affect the identity of reproductive organs [26]. However, a loss-of-function experiment showed that the *zag1* mutation generated indeterminate floral meristems instead of a carpel in the center of the ear [26].

This result is similar to that of the loss-of-function experiment of *ag* and *ple* in dicot plants.

We have reported the isolations of seven MADS box genes from rice. Among them, *OsMADS1* [7], *OsMADS5* [19], *OsMADS7* [18], and *OsMADS8* [18] were classified as members of the *AGL2* gene family, *OsMADS2* [6] and *OsMADS4* [6] as members of the *GLO* gene family and *OsMADS3* [20] as a member of the *AGAMOUS* gene family based on sequence homology. Functional analysis by ectopic expression in a heterologous tobacco system indicated that *OsMADS1*, *OsMADS5*, *OsMADS7*, and *OsMADS8* [18, 19] are involved in controlling flowering time and that *OsMADS3* [20] is important for anther development. Here, we report the results from loss-of-function experiments of *OsMADS3* and *OsMADS4* in rice plants. The transgenic plants exhibited homeotic conversion of floral organs, which indicates that these genes are functionally equivalent to the class B and C genes of dicot plants.

Materials and methods

Bacterial strains

Escherichia coli JM 83 was used as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* LBA4404 [14] containing the *Ach5* chromosomal background and a disarmed helper-Ti plasmid pAL4404 was used for transformation of rice.

Construction of binary vectors

We constructed the binary vector pGA1611 that can be used for transformation of rice plants. This vector, a derivative of pGA482 [1], contains the hygromycin phosphotransferase (*hph*) gene as a selectable marker under the control of the cauliflower mosaic virus 35S promoter followed by the termination region of the 7 gene of pTiA6. The vector also contains several unique sites (*HindIII*, *SacI*, *HpaI*, and *KpnI*) between the maize ubiquitin (*Ubi-1*) promoter, including the first intron of the ubiquitin gene [5], and the nopaline synthase (*nos*) terminator. Therefore, this vector can be used for expression of a foreign gene in monocot plants when transferred by the *Agrobacterium* co-cultivation method. The cDNA clones of *OsMADS3* and *OsMADS4* were inserted into multiple cloning sites in an antisense orientation, constructing pGA1622 and pGA1624, respectively.

Rice transformation

A japonica rice variety, Nackdong, was used for transformation by the *Agrobacterium* co-cultivation method as described previously with the following modifications [13]. Calli were induced from the scutellum of mature seeds on an N6 medium containing 2 mg/l 2,4-D. *A. tumefaciens* carrying either the pGA1622 or pGA1624 plasmid was grown for 3 days in an AB liquid medium supplemented with 15 mg/l hygromycin and 3 mg/l tetracycline. Three-week-old calli were co-cultivated with the *Agrobacterium* on a 2N6-As medium supplemented with 100 μ M betaine for 2–3 days in darkness at 25 °C. The co-cultivated calli were washed with sterile water containing 100 mg/l cefotaxime, and incubated on an N6 medium containing 40 mg/l hygromycin and 250 mg/l cefotaxime for 3 weeks. Actively growing calli were transferred onto a regeneration medium, MS medium supplemented with 0.1 mg/l NAA, 2 mg/l kinetin, 2% sorbitol, 1.6% phytagar (Gibco), 50 mg/l hygromycin B, and 250 mg/l cefotaxime. After 2–3 weeks under continuous light (40 μ mol m⁻² s⁻¹), plantlets were transferred to soil and grown in a growth chamber with 10 h light per day.

RNA blot analysis

Total RNA from leaves of rice plants was isolated by the RNA isolation kit (Tri Reagent, Molecular Research Center, INC). A 20 μ g portion of total RNA was fractionated on a 1.3% agarose gel as described previously [33]. After RNA transfer onto a nylon membrane, the blot was hybridized in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS for 20 h at 60 °C [8]. After hybridization, the blot was washed twice with a solution containing 0.1 \times SSPE and 0.1% SDS for 5 min at room temperature followed by two washes of the same solution at 60 °C for 15 min.

Microscopic techniques

Rice flowers were fixed in 50% ethanol, 0.9 M glacial acetic acid and 3.7% formaldehyde for 15 h at 4 °C, dehydrated with ethanol, infiltrated with xylene and embedded in a paraffin (paraplast x-tra, Oxford labware). Sections 12 μ m thick were attached to gelatin-coated glass slides, deparaffinized in xylene and rehydrated in a graded ethanol and water series. Sections were incubated in 1.0% aqueous safranin O for 6 h, excess stain was rinsed away with tap water, and the

sample dehydrated with ethanol. The sections were stained in 0.5% fast green in 95% ethanol for 40 sec, rinsed in 95% ethanol, infiltrated with xylene, and covered permanently. Light microscopy was performed with a Nikon labophoto-2.

Results

Transformation of rice plants with the antisense OsMADS4 construct

We have previously reported the isolation of a rice cDNA clone, *OsMADS4*, that encodes a MADS box protein [6]. Its amino acid sequences and expression patterns were highly homologous to those of *GLO* and *PI*. In order to elucidate the role of the *OsMADS4* gene in rice, the *OsMADS4* cDNA clone was placed in an antisense orientation under the control of the maize ubiquitin promoter and the *nos* terminator, constructing the binary vector pGA1624. The chimeric molecule was introduced to rice plants using the *Agrobacterium*-mediated transformation method [13]. Forty independent hygromycin-resistant rice plants were generated. All of the transgenic plants showed normal development of vegetative organs and produced panicles. There was no change in the heading time or the height. However, 38 transgenic plants exhibited abnormal phenotypes of reproductive organs, including altered floral organ development and reduced fertility.

RNA blot analysis of the *OsMADS4* transgenic plants

Expression of the transgene was studied using RNA samples prepared from leaves of the transgenic plants. Since the ubiquitin promoter was used to express the transgene, the *OsMADS4* transcript should have been detectable in the transgenic leaves. Among six plants selected for the RNA blot analysis, three showed significant changes in their floral organs, and the other three plants showed less phenotypic changes. The results showed that the A4-4, -14, and -22 plants accumulated higher levels of the *OsMADS4* transcript and the other three plants with milder phenotypes accumulated lower levels of the transcript (Figure 1). The *OsMADS4* transcript was not detected in the control non-transgenic plant leaves.

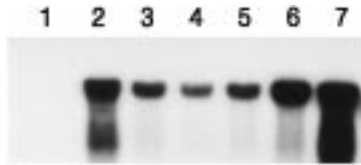


Figure 1. RNA blot analysis of the *OsMADS4* antisense transcript in transgenic rice. RNA was isolated from the mature leaves of a control plant (lane 1) and six different transgenic plants (lanes 2–7). Lanes 2, 6 and 7 represent the *OsMADS4* antisense transcripts in A4-04, -22, and -14, respectively. A 25 μ g portion of total RNA was hybridized with 32 P-labeled DNA prepared from a gene-specific *OsMADS4* DNA probe.

Phenotypic alteration of floral organs in OsMADS4 transgenic plants

The three plants, A4-4, A4-14, and A4-22, that showed the most severe alterations and accumulated higher levels of the transgene transcript were selected for further studies. These lines displayed phenotypic changes in their lodicules, stamens, and carpels (Figure 2). In the A4-4 flowers, two or three anthers were distorted and their filaments were shortened and thickened (Figure 2B). In addition, a stigmatic papillae-like tissue was generated at the tip of the anthers. Lodicules of the flowers became thinner and longer compared to those of a normal flower (Figure 2A). The paleas, lemmas, and carpels of the transgenic plants showed nearly normal phenotypes. The flowers of the A4-14 and A4-22 plants displayed more severe alterations in their stamens compared to the A4-4 flowers. The A4-22 flowers showed alteration in all of their stamens, displaying short, thick, and distorted phenotypes with papillae- or stigma-like tissue at the tips of anthers (Figure 2C). The stamens of the A4-14 flowers exhibited the most severe phenotypic changes, becoming carpel-like organs with one to several stigmas (Figure 2D).

To further analyze the alterations in the flowers, each floral organ of the A4-14 flower was detached from the flower and compared to that of a normal flower (Figure 3). Stamens were deformed into various abnormal shapes that resemble carpels (Figure 3B). Small, fat, and oval lodicules were changed into long and thin organs (Figure 3C). Unlike the severe alterations in anthers and lodicules, the carpel was barely modified, showing a nearly normal stigma, style, and ovary (Figure 3A).

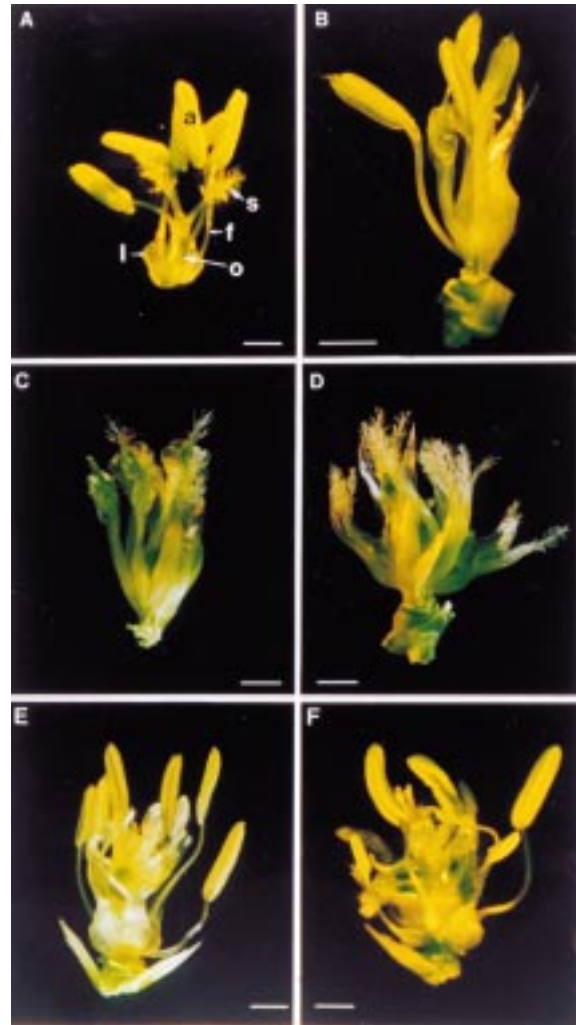


Figure 2. Phenotypic alterations of transgenic rice flowers carrying the *OsMADS4* and *OsMADS3* antisense RNAs. A. Wild-type rice flower. B–D: Flowers of A4-04, A4-22, and A4-14. E, F. Flowers of A3-33. a, anther; f, filament; o, ovary; s, stigma; l, lodicule. Bars = 1 mm.

Histological analysis of the OsMADS4 transgenic flower

To investigate histological modification of the altered organs, a flower of the A4-14 transgenic plant was embedded in paraffin and horizontally sectioned through the lower, middle, and upper parts. In the lower section, one of the filaments exhibited a shape similar to that of the ovary (Figure 4D). The remaining five filaments were increased in size and irregularly deformed. The number of vascular bundles in each transgenic filament increased to up to several whereas the wild-type filaments contain one. In the upper section, each

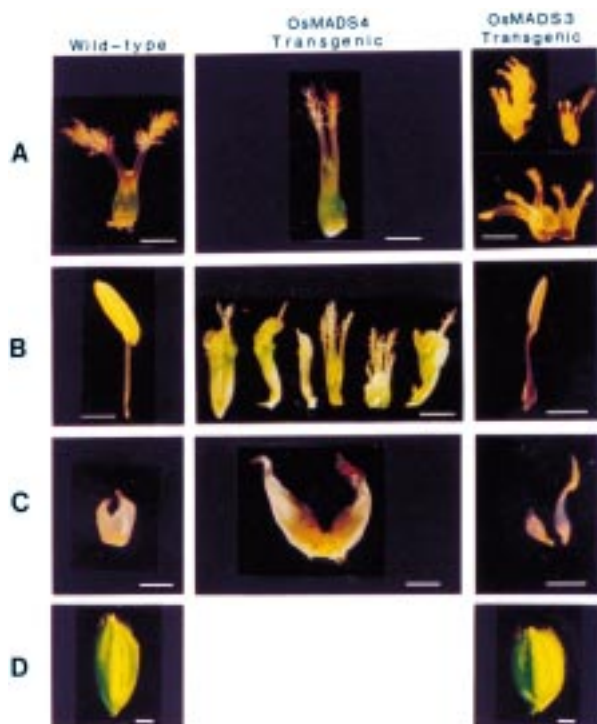


Figure 3. Comparison of each floral organ between wild-type, transgenic A4-14, and transgenic A3-33 flowers. The left panels show the carpel (A), stamen (B), lodicule (C), and palea/lemma (D) of the wild type flower and the two right panels show those of the A4-14 and the A3-33 flower respectively. Bars = 1 mm.

of the six anthers was very much distorted into different shapes and failed to produce mature pollen (Figure 4F). Lodicules of wild type flowers are composed of distinct tissues (Figure 4A and B), but the lodicules of the transgenic plant resembled the palea and lemma (Figure 4D and E).

Transformation of rice plants with the antisense *OsMADS3* construct

We have previously isolated another rice MADS box gene, *OsMADS3*, whose sequence and expression patterns were highly homologous to those of *AG* and *PLE* [20]. It was suggested that the gene belongs to the class C MADS box gene family since ectopic expression of *OsMADS3* in tobacco resulted in phenotypes that resembled those expressed by the *AG* gene and *AG* homologues [20]. In order to study the functional roles of the gene in rice, the *OsMADS3* cDNA clone was placed under the control of the ubiquitin promoter in an antisense orientation. The chimeric molecule was transferred into rice chromosomes using the *Agrobacterium*-mediated transformation method. One



Figure 5. RNA blot analysis of the *OsMADS3* antisense transcript in transgenic rice. RNA was isolated from the mature leaves of a control plant (lane1) and five different transgenic plants (lanes 2–6). Lanes 4 and 5 represent the *OsMADS3* antisense transcripts in A3-33 and -07, respectively. A 25 μ g portion of total RNA was hybridized with 32 P-labeled DNA prepared from a gene-specific *OsMADS3* DNA probe.

hundred hygromycin-resistant plants were obtained and grown to maturity. All of the transgenic plants exhibited normal growth and development during the vegetative growth phase and generated panicles. However, most of the plants bore abnormal flowers and sterile seeds.

RNA blot analysis of the *OsMADS3* transgenic plants

Five independently transformed plants were selected to measure the level of the *OsMADS3* transcript in their transgenic leaves. Two plants, A3-7 and A3-33, showed severe alterations of the floral organs, and the other three plants showed little phenotypic change. The blot analysis showed that the A3-7 and A3-33 plants accumulated higher levels of the *OsMADS3* transcript compared to other three plants that exhibited less phenotypic change (Figure 5).

Phenotypic alteration of floral organs in *OsMADS3* transgenic plants

The A3-7 and A3-33 plants were selected for further studies. In A3-7 flowers, the lower portion of some filaments was enlarged, forming oval, fat, and fleshy organs that resemble the lodicule. In some transgenic flowers, the number of anthers increased to seven or eight, rather than the normal six (data not shown). There were no apparent alterations in the palea/lemma or carpel. Flowers of the A3-33 plant exhibited more severe phenotypic changes, showing alterations in the anthers, carpels, and lodicules (Figure 3E and F). Figure 3 shows each floral organ of A3-33 in comparison with the wild type flower. Figure 4 shows transverse sections of the transgenic flower at three positions. These figures indicate that the fourth whorl consists of several abnormal flowers with undifferentiated stamens and carpels in the transgenic plant instead of a normal carpel. This phenotype resembles the flowers of transgenic *Arabidopsis* plants expressing antisense

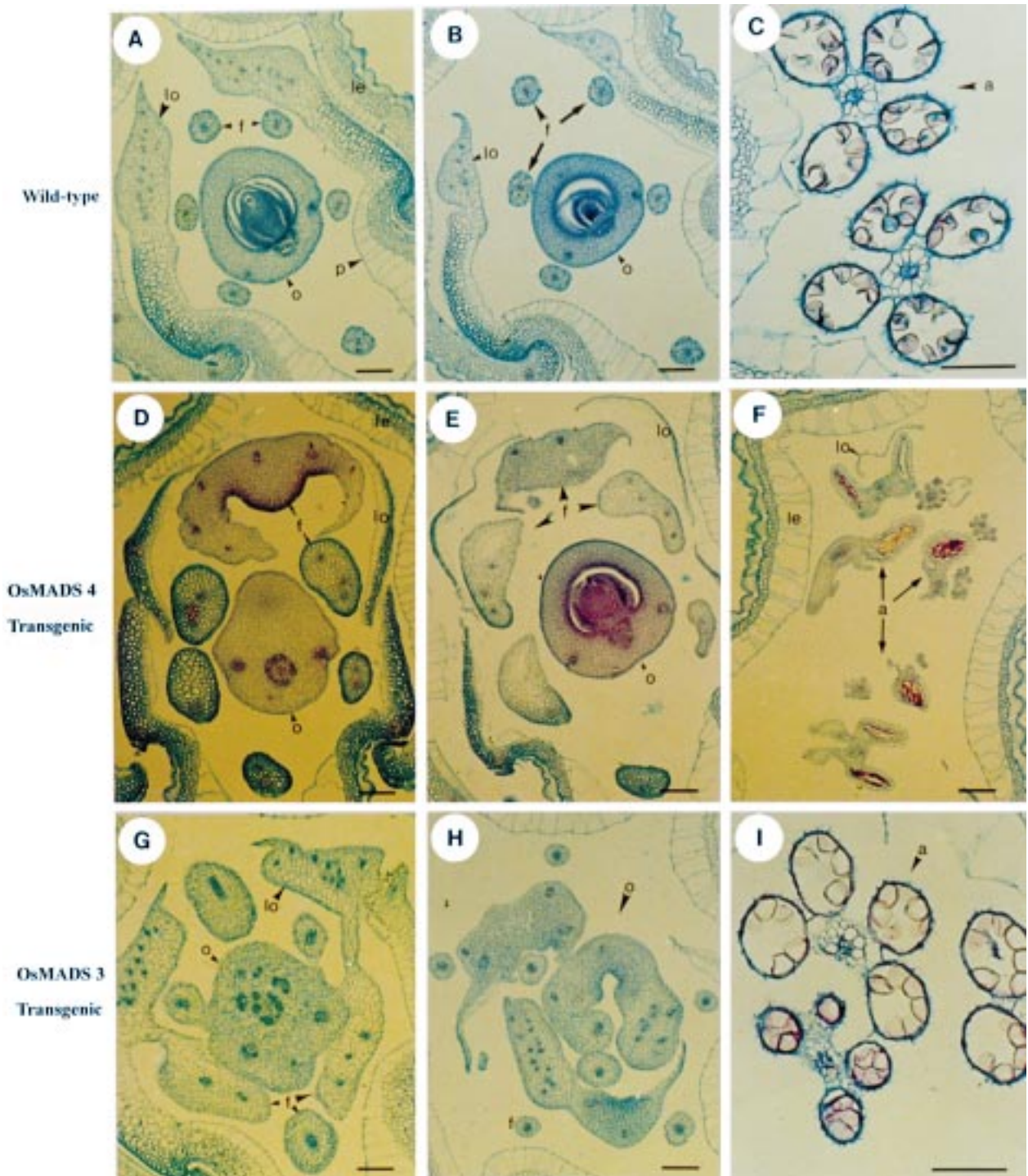


Figure 4. Sections of the mature flowers of wild-type, transgenic A4-14, and A3-33 plants. The upper panels show horizontal sections of the lower (A), middle (B), and upper (C) parts of the wild-type rice flower, the middle panels show those of the A4-14 flower and the lower panels show those of the A3-33 flower. p, palea; le, lemma; f, filament; a, anther; o, ovary; lo, lodicule. Bars = 100 μ m

AG RNA [28]. The stamens of the A3-33 flower were occasionally altered; the filament base was changed into a lodicule-like structure, and anthers became smaller than the wild type (Figures 2F and Figure 3B). The transverse sections of the flower at the basal part showed that shape and size of the filaments were different from one another, and some filaments were converted into a lodicule-like structure (Figure 4G). The transverse sections through the transgenic anthers revealed that both the small and normal sized anthers generated pollen grains (Figure 4I). Lodicules of the transgenic plant were changed into several types of unusual organs, including stamen-like structures (Figure 3C). This phenotype does not match the typical ABC model. A simple explanation for the alteration is that *OsMADS3* may be involved in the formation of lodicule in addition to the typical C function in rice. The palea and lemma, the outermost organs of rice flower, became shorter and fatter compared to the wild type (Figure 3D).

Discussion

We have studied the roles of two rice MADS box genes by the loss-of-function approach. Antisense expression of *OsMADS4* resulted in homeotic alterations of the second and third whorls. The second whorl lodicules were changed to palea/lemma-like structures, and the third whorl stamens were changed to carpel-like organs. This result supports that *OsMADS4* is a member of the class B family. The class B organ identity genes have been extensively studied from several dicot species. Two class B genes are known for both *Arabidopsis* (*AP3* and *PI*) and *Antirrhinum* (*DEF* and *GLO*). For all four class B genes, transcripts are initially detected in young flower primordia, but there are differences in their spatial distribution. The *PI* and *DEF* genes are transcribed in the second, third, and fourth whorls, whereas transcripts of the *AP3* and *GLO* are limited to the second and third whorls [32]. Although the spatial expression pattern of the *PI* and *DEF* genes are similar to each other, they are not considered to be orthologous genes since their amino acid sequence similarity is low: *PI* is closely related to *GLO* (58% identity) and *DEF* is similarly related to *AP3* (61% sequence identity) [32]. In addition, *DEF* could functionally replace *AP3* in making petals and stamens [32]. The *OsMADS4* protein is most homologous to *GLO* (54%) and *PI* (51%), and the homology was much lower with *AP3* (35%) and *DEF* (32%) [6].

However, the expression pattern of *OsMADS4* was more similar to *DEF*, since the *OsMADS4* transcript is present in the fourth whorl [6].

The antisense effects of the *OsMADS4* gene were restricted to the second and third whorls, and the first and fourth whorls appeared normal. Similar phenotypes were observed from the *Arabidopsis* and *Antirrhinum* mutants defective in the function of class B genes. Loss-of-function analyses of the class B MADS box genes by the antisense approach have not been previously reported. Instead, it was observed that cosuppression of petunia class B genes, *FBPI* and *pMADS1*, also resulted in homeotic alterations of the second and third whorls [2, 3, 38]. Therefore, the function of class B organ identity genes appears to be broadly conserved between dicot and monocot plants.

Flowers of the grass family have evolved distinctively apart from those of dicots. In rice, the unit of inflorescence is the spikelet, which bears the lemma, palea, two lodicules, six stamens, and carpel [41]. Lodicules are small, oval, thick, and fleshy organs located at the base of the ovary and are considered to be petals [41]. However, it is uncertain whether the lemma and palea are equivalent to sepals. In this study, it was observed that antisense expression of the *OsMADS4* gene caused the lodicule to change so that it resembled the palea/lemma. In dicot plants, an alteration of petals toward sepals is a typical phenotype in the mutants that have lost the class B organ identity genes [3, 10, 11, 16, 23, 32, 35, 36]. Therefore, the observations are consistent with the hypothesis that the palea/lemma and sepals have a common ancestry.

The *AG* gene from *Arabidopsis* and the *PLE* gene from *Antirrhinum* are the class C organ identity genes that control development of stamens and carpels. We have shown previously that the rice *OsMADS3* gene is an *AG* homologue. Both genes share similarities in amino acid sequences, expression patterns, and effects of ectopic expression [20]. In this study, we have provided further evidence that the *OsMADS3* gene is a class C organ identity gene. The transgenic plants expressing the antisense *OsMADS3* transcript produced abnormal flowers and sterile seeds. Flowers of these plants showed homeotic alterations in their carpels and stamens. In the fourth whorl, the carpel was replaced by several abnormal flowers with undifferentiated stamens and carpels. The third whorl stamen was changed into a lodicule-like structure. Such alterations in the inner two whorls of the flower are similar to the phenotypes of *Arabidopsis ag* mutants and *Antirrhinum ple* mutants [39]. Similar al-

terations were previously observed in dicot plants by the loss-of-function approach. The flowers of transgenic *Arabidopsis* plants carrying antisense *AG* RNA carried indeterminate floral meristems and homeotically converted reproductive organs, showing the same phenotypes as the *ag* mutant [28]. Flowers of the transgenic tomato plants that express *TAG1* antisense RNA displayed homeotic conversion of third whorl stamens into petaloid organs and the replacement of fourth whorl carpels with pseudocarpels bearing indeterminate floral meristems with nested perianth flowers [31]. Tobacco plants that express the *NAG1* cDNA in the antisense orientation showed a partial conversion of stamens into petals, but the carpels of the transgenic plants were not converted into new flowers, unlike the carpels of other plants transformed by antisense *AG* genes [22]. This was probably due to the fact that the amount of antisense transcript was insufficient to suppress endogenous *NAG1* activity [18]. Mena *et al.* [22] reported identification of two class C organ identity genes, *ZAG1* and *ZMM2*, from maize. The *ZAG1* RNA was preferentially accumulated in the carpel, whereas the *ZMM2* RNA was in stamens. Unlike other organisms, *ZAG1* mutants exhibited alteration of only carpel development, suggesting that *ZAG1* and *ZMM2* share some redundancy in function.

In summary, we have presented that *OsMADS4* and *OsMADS3* are class B and C organ identity genes, respectively. Our results indicate that the functions of the class B and C MADS box genes have been highly conserved during evolution between monocots and dicots, despite the distinct differences in their flower morphology.

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