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Morphological alterations by ectopic expression of the rice *OsMADS4* gene in tobacco plants

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Abstract *OsMADS4*, a rice MADS-box gene, is a member of the *GLO/PI* family that specifies the identity of petals and stamens in combination with other MADS-box genes. We report here the ectopic expression of *OsMADS4* fused to the CaMV 35S promoter in tobacco plants. Transgenic plants carrying the CaMV 35S promoter::*OsMADS4* construct generated mutant flowers with a mosaic carpel, in which the tissue around the nectary was elongated and the styles reduced. The fruits were distorted, but viable seeds did develop. These phenotypes mimicked those of transgenic tobacco plants that ectopically express *Antirrhinum GLO*. However, unlike *GLO*, *OsMADS4* did not cause any homeotic change in the first whorl of the transgenic flowers. These results suggest that the functional role of *OsMADS4* in the outer whorls has diverged from that of its dicot counterparts.

Keywords Flower development · *GLO/PI* · MADS box · Rice

Abbreviations *CaMV*: Cauliflower mosaic virus · *MADS*: MCM1, AGAMOUS, DEFICIENS, and Serum responsible factor

Introduction

In most dicot angiosperms, the floral organs develop on four different whorls consisting of the sepal, petal, sta-

men, and carpel. However, flowers of monocot plants have evolved separately from those of the dicots (Ma and de Pamphilis 2000). The flowers of most Liliaceae have two outer whorls of almost identical petaloid organs, called tepals, instead of the sepal and petal whorls of the dicots (Kanno et al. 2003). In rice, one of the grasses, the unit of inflorescence (panicle) is the spikelet, which bears a floret along with the palea/lemma, two lodicules, six stamens, and a carpel. The lodicules on the second whorl are morphologically and functionally distinctive from the petals and tepals.

According to the ABC model, the B-class homeotic genes determine the fate of the second and third whorls. These genes work with A-class genes to specify second-whorl organ identity and with C-class genes to determine the fate of the third-whorl organs (Lohmann and Weigel 2002). For example, *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum majus* (Sommer et al. 1990; Trobner et al. 1992) and *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis thaliana* (Jack et al. 1992; Goto and Meyerowitz 1994) belong to the B class. These genes encode regulatory proteins sharing the MADS box, a DNA-binding motif (Sommer et al. 1990). In addition to the DNA-binding domain, plant MADS genes share a conserved motif called the K box, which plays an important role in protein-protein interaction (Ma et al. 1991; Pnueli et al. 1991).

Analyses of ectopic expression have been performed to study the function of B-class genes in several plant species. Flowers from transgenic lines that constitutively express *AP3* under the control of the 35S promoter exhibit the replacement of carpels by stamens, while the sepals remain unaffected (Jack et al. 1994). Ectopic expression of both *AP3* and *PI* result in the transformation of the carpels into stamens and the sepals into petals (Krzek and Meyerowitz 1996). Heterologous ectopic expression analyses have also been used to predict functional roles for B-class MADS-box genes. For example, expression of the *GLO* gene in tobacco causes homeotic transformation of the carpels into stamen-like organs and sepals into petaloid organs (Davies et al. 1996). The expression of truncated *LMADS1*, the *AP3* homolog in a monocot lily (*Lilium longiflorum*), in

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Arabidopsis generates the *ap3*-like dominant negative mutation, in which the petals are converted into sepaloid organs and stamens into carpeloid organs (Tzeng and Yang 2001). Finally, constitutive expression of the *OMADS3* gene, an *AP3*-like gene in orchid, in *Arabidopsis* causes the development of terminal flowers similar to those observed in transgenic plants ectopically expressing A-class genes such as *API* (Hsu and Yang 2002).

The B-class genes have also been intensively studied in cereal species—for example, rice and maize (Munster et al. 2001). Loss-of-function mutations of *OsMADS4*, a *GLO/PI*-like gene, in transgenic rice plants and of *SILKY1*, the *DEF/AP3*-like gene, in maize causes homeotic transformations of lodicules to palea-like structures and stamens to carpeloid organs (Kang et al. 1998; Ambrose et al. 2000). Ectopic expression of *OsMADS16*, the *DEF/AP3*-like gene, in transgenic rice plants results in the homeotic alteration of carpels into stamenoid carpels (Moon et al. 1999; Lee et al. 2003). All of these findings indicate that the ABC model can be applied to rice and maize. However, unlike *Antirrhinum* and *Arabidopsis* where the *GLO/PI* gene is unique, rice has two *GLO/PI*-like genes—*OsMADS2* and *OsMADS4* (Chung et al. 1995)—while maize has three. One of those maize genes, *ZMM16*, is most similar to *OsMADS2*, whereas the other two, *ZMM18* and *ZMM29*, are homologous to *OsMADS4* (Munster et al. 2001).

Loss-of-function analysis of the *OsMADS2* gene using the double-stranded (ds)RNAi method has shown that this gene functions to determine the fate of the second whorl (Prasad and Vijayraghavan 2003). This suggests that duplication of the rice *GLO/PI*-like genes could be related to their functional diversification of the first- and second-whorl organs in cereal plants. In the study reported here, we demonstrated that ectopic expression of rice *OsMADS4* in tobacco plants generated a homeotic alteration of carpels without any change in the first whorl, thereby supporting the possibility that the rice *GLO/PI*-like gene has diverged from that of dicot species.

Materials and methods

Bacterial strains, plant materials, and plant transformation

Escherichia coli strain JM83 served as the recipient for routine cloning experiments. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983), containing the *Ach5* chromosomal background and a disabled helper-Ti plasmid, pAL4404, was used for the transformation of tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) by the co-cultivation method (An et al. 1988). Transgenic plants were maintained under standard greenhouse conditions.

Construction of chimeric molecules

We have previously reported the sequences of *OsMADS2* and *OsMADS4* cDNAs, including their 5' and 3' untranslated regions (Chung et al. 1994, 1995). Each cDNA was inserted in the sense orientation into the *EcoRI* site of pGA748, a derivative of pGA643 (An et al. 1988), resulting in the construction of pGA1210 (CaMV35Sp::sense *OsMADS2*) and pGA1352 (CaMV35Sp::sense *OsMADS4*).

RNA gel-blot analysis

Total RNA was isolated from transgenic tobacco plants by the guanidium thiocyanate method. Aliquots (20 μ g) of total RNA were fractionated on a 1.3% agarose gel as described by Kang and An (1997). Following RNA transfer onto a nylon membrane, the blot was hybridized for 20 h at 60°C in a solution containing 0.5 M NaPO₄ (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, and 7% sodium dodecyl sulfate (SDS) (Kang and An 1997). Following hybridization, the blot was washed twice with a solution containing 0.1× SSPE and 0.1% SDS for 5 min at room temperature followed by two washes in the same solution at 60°C for 15 min.

Microscopic techniques

Tobacco flowers were fixed in 50% ethanol, 0.9 M glacial acetic acid, and 3.7% formaldehyde for 15 h at 4°C, then dehydrated with ethanol, infiltrated with xylene, and embedded in paraffin (Paraplast X-tra, Oxford Labware, St. Louis, Mo.; <http://www.kendall-hg.com>). Afterward, 12- μ m sections were attached to gelatin-coated glass slides, deparaffinized in xylene, and rehydrated in a graded ethanol and water series. The sections were incubated in 1.0% aqueous safranin O for 6 h, excess stain was rinsed away with tap water, and the samples dehydrated with ethanol. The sections were stained in 0.5% fast green in 95% ethanol for 40 s, rinsed in 95% ethanol, infiltrated with xylene, and covered permanently (Kang et al. 1998). Light microscopy was performed with a Nikon Labphoto-2 (Nikon, USA; <http://www.nikonusa.com>).

Results

Ectopic expression of *OsMADS4* and *OsMADS2* cDNAs in tobacco

Twenty transgenic tobacco plants carrying the 35S promoter::*OsMADS4* chimeric molecule were generated, and transgene expression was measured using RNA samples prepared from leaves of these plants (Fig. 1a). Because the CaMV 35S promoter was used to express the transgene, we expected the *OsMADS4* transcript to be de-

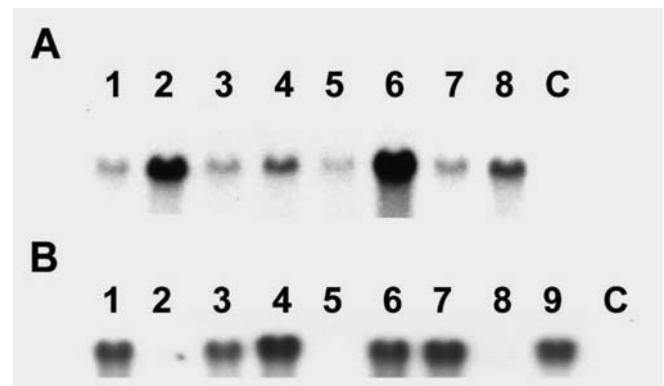
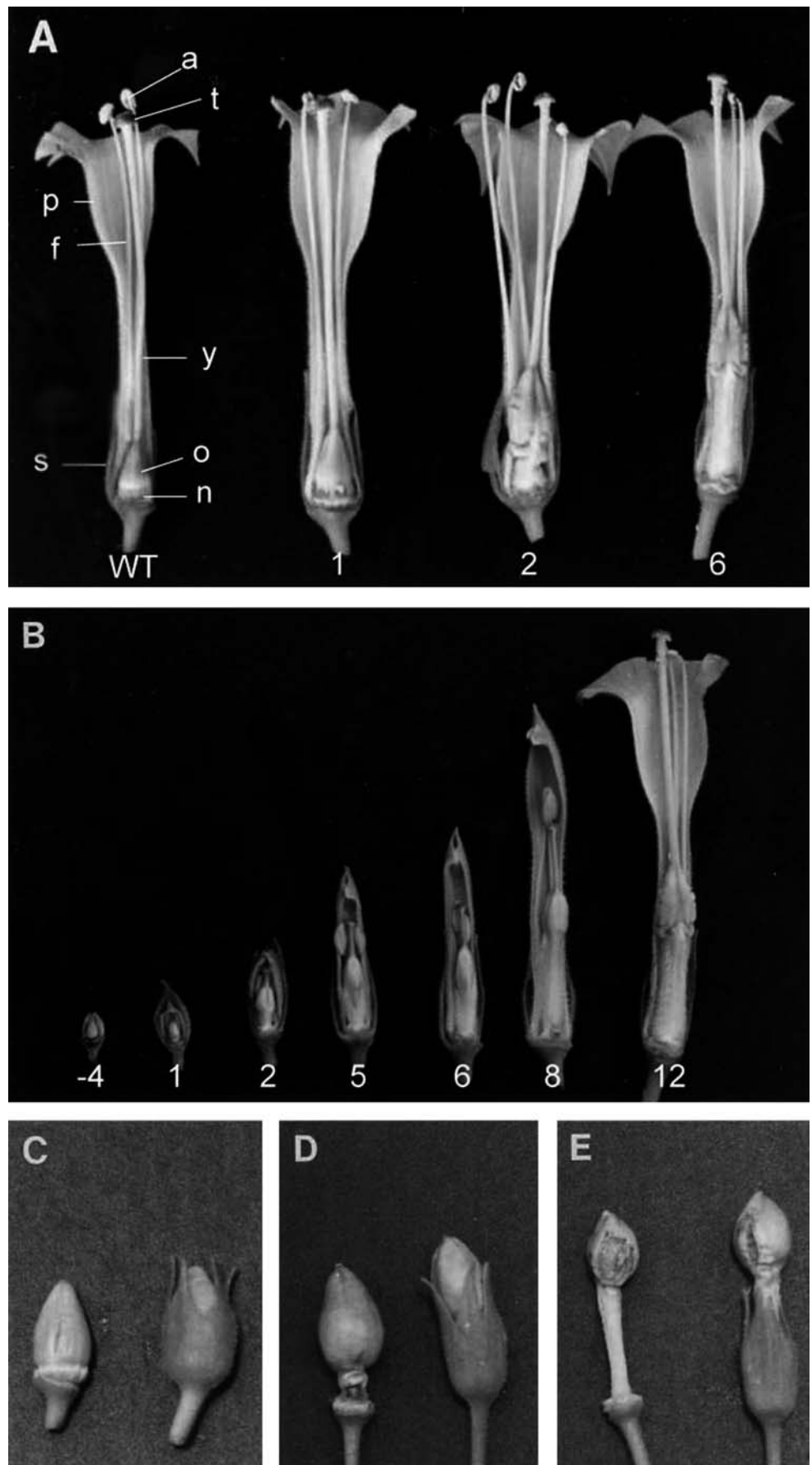


Fig. 1 RNA gel-blot analyses of *OsMADS4* and *OsMADS2* transcripts in transgenic tobacco. **a** RNA was isolated from mature leaves of a control plant (C) and eight CaMV35Sp::*OsMADS4* transgenic plants (1–8) exhibiting altered phenotypes with respect to the female organs. **b** RNA from leaves of control plant (C) and nine *OsMADS2* transgenic plants (1–9). Twenty micrograms of total RNA was hybridized with [³²P]-labeled DNA probes prepared from gene-specific regions of *OsMADS4* and *OsMADS2*

Fig. 2 Phenotypes of transgenic tobacco plants expressing *OsMADS4*. **a** Transgenic flowers showing abnormal floral morphology. *Left* Wild-type (WT) tobacco flower, *right* flowers from transgenic plants [numbers (1, 2, 6) indicate transgenic lines shown in Fig. 1]. *s* Sepal, *a* anther, *t* stigma, *p* petal, *f* filament, *y* style, *o* ovary, *n* nectary. **b** Flower development of plant no. 6. Numbers indicate developmental stages. **c–e** Phenotypes of seed pods from wild-type (c) and transgenic plant nos. 2 (d) and 6 (e)



tectable in the transgenic leaves but not in those of the untransformed control plants (Fig. 1). Among the eight transgenic plants selected for RNA blot analysis, plant nos. 2 and 6, with severe phenotypes, accumulated higher levels of the *OsMADS4* transcript while, in contrast, transgenic lines with weak phenotypes had lower levels. This result demonstrates that the abnormality in the fourth whorls was proportional to the degree of *OsMADS4* ectopic expression.

The fourth whorl of a wild-type tobacco flower has two fused carpels that can be subdivided into a cone-shaped, green ovary at the base and a long style with a broadly rounded stigma (Fig. 2a). Among the 20 transgenic plants, eight exhibited weak phenotypes, i.e., pale-green and swollen ovaries, and brownish nectaries (Fig. 2a, no. 1). Seven plants showed intermediate phenotypes with pale-green, swollen, and wrinkled ovaries, and shortened styles (Fig. 2a, no. 2). The other five plants had strong phenotypes, in which the nectaries were severely elongated (Fig. 2a, no. 6). The bases of those nectaries were covered with hair-like structures, and the anthers were positioned below the stigmas.

We selected transgenic plant no. 6 to observe structural alterations to floral organs over time. Development of a wild-type tobacco flower can be divided into 19 stages—seven stages of pre-meiosis and 12 stages of post-meiosis (Koltunow et al. 1990). At Stage 1 (8-mm floral buds), the flowers of plant no. 6 did not differ distinctly from those of the wild-type (Fig. 2b). At Stage 2 (11-mm floral buds), however, the base of the ovary of transgenic plant no. 6 started to elongate, and at Stage 5 (about 20-mm floral buds), hairy structures were formed on the surface of the abnormally elongated organ. Finally, at the mature flower stage, the stigma was located above the anthers (Fig. 2b).

Following flowering, the seed pod of plant no. 6 developed poorly, and its surface was rotten. Despite those distortions, all of the transgenic plants produced fertile seeds (Fig. 2d,e). These phenotypes are similar to those of the 35S::*GLO* transgenic tobacco plants reported by Davies et al. (1996). However, unlike with *GLO*, *OsMADS4* did not cause any homeotic change in the first whorl of our transgenic plants.

In contrast, 20 transgenic plants carrying the 35S promoter::*OsMADS2* chimeric molecule did not show any phenotypic alteration in their floral organs, even though the transgene was being expressed (Fig. 1b). We have also co-expressed *OsMADS2* and *OsMADS4* in tobacco by crossing the transgenic plants over-expressing *OsMADS2* and *OsMADS4*. However, we did not find any new morphological changes compared to the transgenic plants over-expressing *OsMADS4* alone.

Microscopic analysis of the transgenic plant

To investigate any histo-modifications in the altered structure, the female organs and seed pods of transgenic plant no. 6 were embedded in paraffin and sectioned

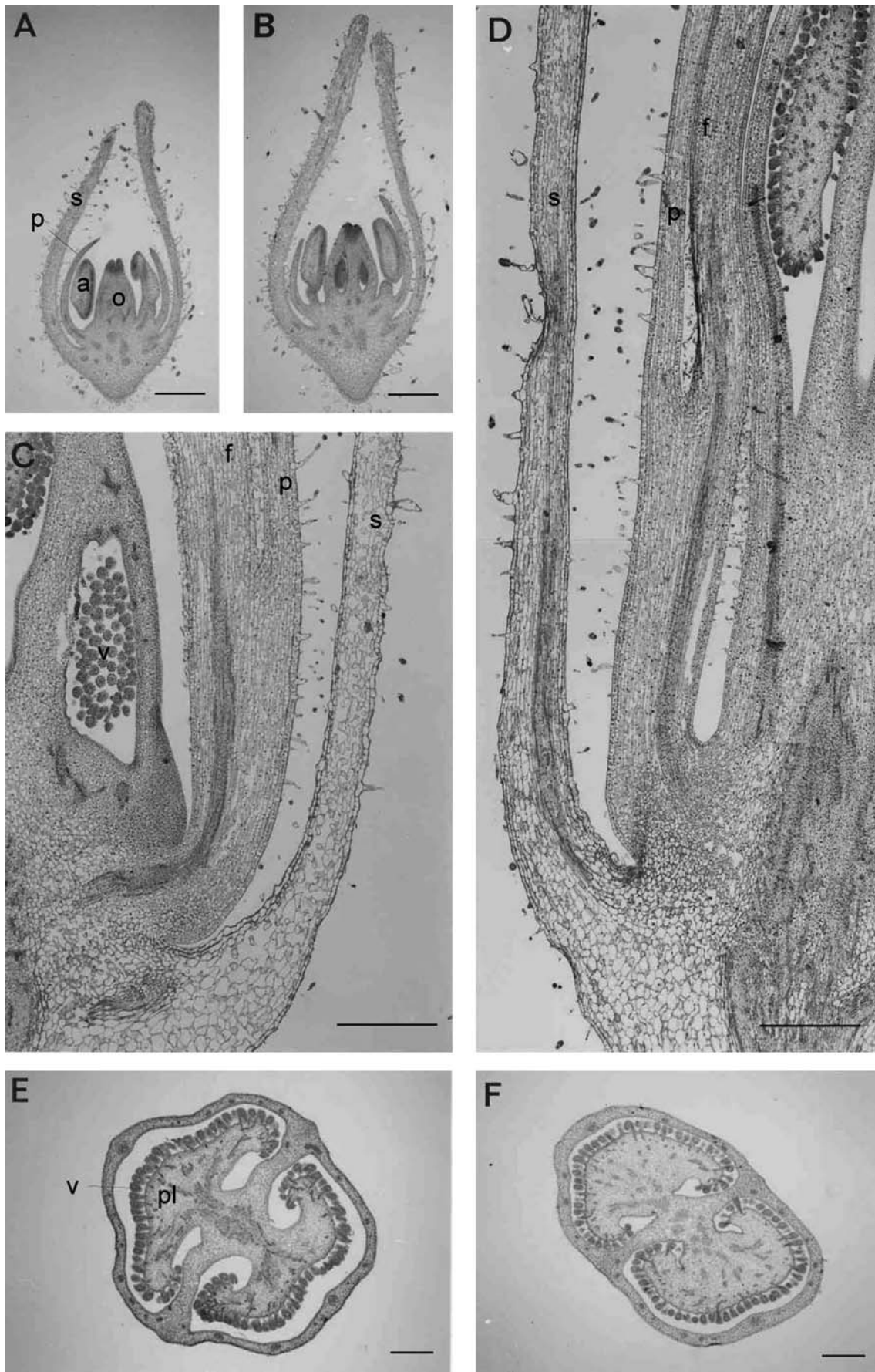
vertically or horizontally. No significant difference was observed between the wild-type flower and transgenic flowers at Stage 1—i.e., those having an 8-mm floral bud (Fig. 3a,b). At Stage 3 (about 14-mm floral bud), however, the tissues below the ovary were elongated in the transgenic plant (Fig. 3d) relative to those of the wild-type flower (Fig. 3c). This lengthening occurred at a time when the filaments were elongating. Nevertheless, although the ovary tissue was distorted, the ovules developed normally (Fig. 3f).

Discussion

Rice has two genes, designated *OsMADS2* and *OsMADS4*, which belong to the *GLO/PI* gene family (Chung et al. 1995). They show 83% amino acid homology to each other and approximately 50% similarity with *GLO* or *PI*. In loss-of-function studies using antisense or dsDNAi approaches, it has been demonstrated that *OsMADS4* is responsible for specifying the fate of the second and third whorls (Kang et al. 1998), while *OsMADS2* determines the fate of the second whorl specifically (Prasad and Vijayraghavan 2003). Here, we report the effects of ectopic expression of *OsMADS4* and *OsMADS2* in the heterologous tobacco plant system.

When *OsMADS4* or *OsMADS2* full-length cDNA was over-expressed under the 35S promoter, the resulting transgenic rice plants did not show any floral homeotic transformation (data not shown). Figure 4 illustrates where the B-function genes are expressed in tobacco and rice and how they affect flower development in the transgenic plants when *OsMADS4* or *OsMADS2* is expressed ectopically. In the ABC model, heterodimerization of *DEF/AP3* and *GLO/PI* is critical to the completion of the B-function gene specifying the petal and stamen (Ma and de Pamphilis 2000). Since expression of *OsMADS16*, an ortholog of *DEF/AP3*, is restricted to the second and third whorls, *OsMADS2* or *OsMADS4* might not have a chance to meet *OsMADS16* in the first and forth whorls of the flowers of transgenic rice plants ectopically expressing *OsMADS2* or *OsMADS4* (Fig. 4b). On the other hand, in transgenic rice plants over-expressing *OsMADS16*, the innermost-whorl carpels were replaced by stamen-like organs (Lee et al. 2003). This alteration was probably due to the formation of a functional complex between the ectopically expressed *OsMADS16* and endogenously present *OsMADS4* in the whorl.

Expression of the *OsMADS4* gene in the tobacco plant caused alterations only to the carpel. If *OsMADS4* were functionally identical to the *PI* MADS genes of dicots, transgenic tobacco plants ectopically expressing the rice *GLO/PI*-like gene should have shown homeotic changes in the first whorl. Because *NTDEF* of tobacco is expressed in all four whorls (Fig. 4c), *OsMADS4* could form a heterodimer with *NTDEF* in the first whorl of the transgenic tobacco flower, thereby causing the sepals to turn into petaloid structures. When *GLO*, the *PI* ortholog in *Antirrhinum*, is expressed ectopically in tobacco, the transgenic



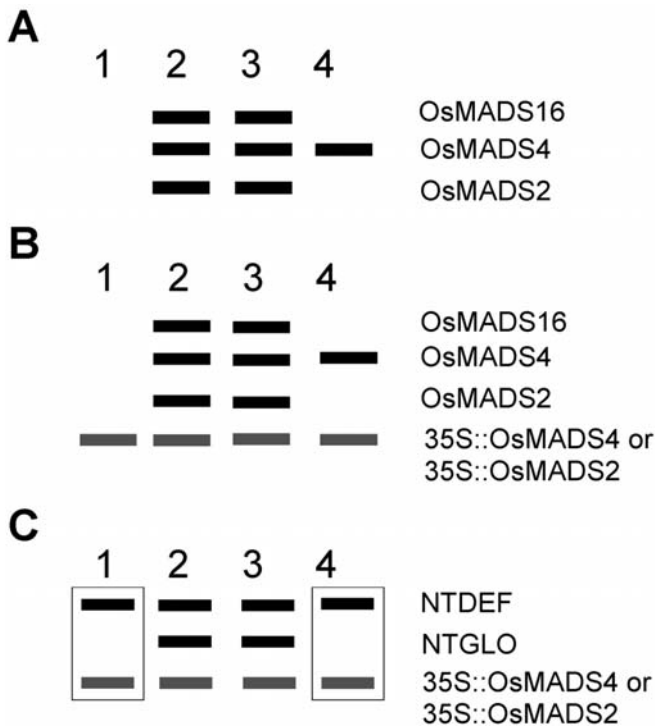


Fig. 4 Illustrative diagram showing the expression pattern of the B-function genes in transgenic rice and tobacco plants over-expressing *OsMADS4* or *OsMADS2*. **a**, Wild-type rice plant, **b** transgenic rice plant, **c** transgenic tobacco plant. Numbers (1–4) indicate each whorl, boxes show the possibility of heterodimerization between NTDEF and *OsMADS4* or *OsMADS2* in whorls 1 and 4

plants produce petaloid sepals in the first whorl and stamenoïd carpels in the fourth whorl, as expected (Davies et al. 1996). Moreover, when *PI* is expressed ectopically in the homologous *Arabidopsis* plant system using the 35S promoter, the transgenic plants exhibit homeotic transformation of the carpels into stamens and the sepals into petals (Jack et al. 1994; Krizek and Meyerowitz 1996). Therefore, our result suggests the possibility that *OsMADS4* does not function in the development of petals, even though it does specify the fate of the second whorl in rice. In fact, true petals are not present in the rice floret. Instead, the second whorl is occupied by the lodicule (Kang et al. 1998). Because *OsMADS2* plays an important role in specifying the second whorl in rice (Prasad and Vijayraghavan 2003), we believe it is also possible that co-expression of *OsMADS2* and *OsMADS4* is needed for homeotic conversion of the first whorl in tobacco.

Fig. 3 Histological analysis of wild-type (**a,c,e**) and transgenic plant no. 6 flowers (**b,d,f**). **a–d** are longitudinal sections, and **e** and **f** transverse sections. **a,b** Stage 4 (4-mm floral bud), **c,d** Stage 3 (14-mm flower), **e,f** mature ovaries. *o* Ovary, *v* ovule, *p* petal, *s* sepal, *f* filament, *pl* placenta. Bars:0.5 mm

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