Note

Double-Stranded RNA Interference of a Rice *PI/GLO* Paralog, *OsMADS2*, Uncovers Its Second-Whorl-Specific Function in Floral Organ Patterning

Kalika Prasad and Usha Vijayraghavan[1]

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

Manuscript received August 16, 2003
Accepted for publication September 2, 2003

ABSTRACT

Unlike many eudicot species, grasses have duplicated *PI/GLO*-like genes. Functional analysis of one of the rice *PI/GLO* paralogs, *OsMADS2*, is reported here. Our data demonstrate its essential role in lodicule development and implicate the second *PI/GLO* paralog, *OsMADS4*, to suffice for stamen specification. We provide the first evidence for differential contributions of grass *PI/GLO* paralogs in patterning second- and third-whorl floral organs.

SPECIFICATION of petals (second whorl) and stamens (third whorl) in eudicot flowers requires a pair of related genes. In Arabidopsis, these genes are *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) and in *Antirrhinum majus* they are *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*). Mutations in either of these pairs of "B-function" genes have similar homeotic effects in the second and third whorls (Lohmann and Weigel 2002). These genes work with "A-function" genes to confer second-whorl organ identity and with "C-function" genes to specify third-whorl identity. Both B- and C-function genes require yet another group of genes, *SEPALLATA* (*SEP1*, *SEP2*, and *SEP3*), for their activity (Lohmann and Weigel 2002). B-function genes have been identified in the monocot cereal grasses, maize and rice, which have highly derived floral organs in the first and second whorls. The latter genes are similar to their eudicot counterparts in that they are expressed in lodicules (second whorl) and stamens (third whorl) and their limited mutational analysis (*silky1* of maize and *spw1* of rice) reveals second- and third-whorl organ identity changes (Ambrose *et al.* 2000; Kyozuka *et al.* 2000; Nandi *et al.* 2000; Munster *et al.* 2001; Nagasawa *et al.* 2003). As in Arabidopsis and Antirrhinum, a single *AP3/DEF* ortholog exists in diverse monocots such as lily (*Lilium regale*), wheat (*Triticum aestivum*), maize, and rice (Munster *et al.* 2001). In contrast, *PI/GLO*-like genes are duplicated in these monocot species (Munster *et al.* 2001). A significant issue to be addressed is whether gene duplication of *PI/GLO*-like genes in grasses has any functional importance. To elucidate the specific function of *OsMADS2*, one of the rice *PI/GLO*-like genes, we have created knockdown phenotypes by exploiting double-stranded RNA-mediated interference, an efficient method to silence genes of interest (Baulcombe 2002). Thirteen independent transgenic lines were generated after transformation with the plasmid pUbi-dsRNAiOsMADS2 (Figure 1A). The floral phenotypes in these transgenic plants were analyzed.

Rice flowers, also called spikelets, are unique in their architecture. Male and female reproductive organs occupy positions identical to those in primitive flowers (Irish 2001). The morphology of organs that surround the reproductive structures differs radically. The eudicot sepals and petals are replaced in rice florets by the lemma/palea and lodicules, respectively (Irish 2001). Additionally, small bracts, termed outer glumes, subtend the spikelet. The gross morphology of flowers in pUbi-dsRNAiOsMADS2 transgenic plants resembles wild-type flowers, particularly with regard to the outer glumes, lemma, and palea (Figure 1, B and C). On the other hand, the second-whorl lodicules (Figure 2F) are approximately three times larger than the small and fleshy wild-type lodicules. The latter are usually wider at the base and narrower at the apex (Figure 2A). The larger transgenic lodicules are also wide at the base, but their significantly greater apical growth produces a structure mimicking the more peripheral organs (Figure 2F). These overgrown second-whorl structures have a green midvein, a feature typical of the outer glume and lemma/palea (Figure 1, B and C) but normally absent in lodicules.

The epidermal cell surfaces of sterile whorl organs in rice flowers are distinctive (Prasad *et al.* 2001). We therefore carried out scanning electron microscopy

[1]*Corresponding author:* Department of Microbiology and Cell Biology, Indian Institute of Science, C. V. Raman Ave., Bangalore 560012, India. E-mail: uvr@mcbl.iisc.ernet.in

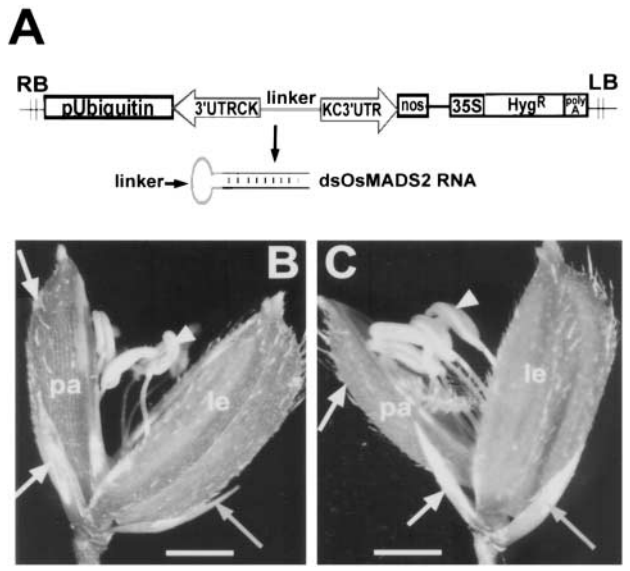

FIGURE 1.—(A) Schematic of transgene construct pUbi-dsRNAiOsMADS2. Transcripts generated can create double-stranded RNA molecules with a loop. LB, the left border; RB, right border; T-DNA repeats flank the Hyg^R and dsRNAiOsMADS2 expression cassettes. K and C represent K-box and C-terminal sequences, respectively. Embryogenic rice calli were transformed with this plasmid and transgenic plants were regenerated as described in PRASAD *et al.* (2001). (B and C) Floral phenotypes of pUbi-dsRNAiOsMADS2 transgenic spikelets. Partially opened wild-type (B) and transgenic rice floret (C) showing outer glume (shaded arrows); le, lemma; pa, palea. Stamens are marked by an open arrowhead; open arrows point to the midvein in the glume and palea. Bar, 1 mm.

(SEM) of the abaxial and adaxial cell surfaces of the transformed lodicules to establish their identity in pUbi-dsRNAiOsMADS2 transgenic plants. The epidermal cells of the wild-type outer glume are arranged in long smooth files (Figure 2, E and K). Cells with rounded projections and trichomes characterize the palea abaxial surface (Figure 2B), while the adaxial surface has smooth, wide, rectangular cells (Figure 2L). Although the epidermal cell morphology of palea is similar to that of the lemma, it can be distinguished from the lemma by a unique marginal tissue that is smooth and trichomeless (Figure 2, A, F, M, and N), rather like the cells of the outer glumes (Figure 2, E and K). Wild-type lodicules have an interlocking pavement-like arrangement of small, compact, rectangular cells (Figure 2, C, D, and O). Epidermal cells of the transgenic outer glume and palea are similar to wild type (compare Figure 2, B and E, with Figure 2, G and J). Strikingly, the enlarged lodicule-like organs of transgenic flowers have characteristics of the outer glume or the palea marginal tissue (Figure 2, H, I, and P–R). The proximal/basal region of these second-whorl organs is a mosaic of cells in elongated files together with cells with some lodicule features (Figure 2, I, P, and Q). The distal/apical portion of these enlarged structures consists of cells morphologically identical to the wild-type outer glume or the palea marginal tissue (compare Figure 2, H and R, with Figure 2, E, K, M, and N). Thus, knockdown of *OsMADS2* perturbs the lodicule differentiation, creating cell types present in more peripheral floral organs. Although *OsMADS2* is expressed throughout stamen primordia specification and differentiation (KYOZUKA *et al.* 2000; NANDI *et al.* 2000), loss of its expression has no effect on stamen cellular differentiation (data not shown). The six stamens and single central carpel of these flowers are normal since the transgenic spikelets were fertile.

To determine early developmental effects of *OsMADS2* knockdown on second-whorl organ patterning, spikelets undergoing organogenesis were taken up for SEM (Figure 3). The initiation of floral organ primordia in these transgenic spikelets is indistinguishable from that of the wild type (Figure 3, A *vs.* D). The lemma and palea primordia are the earliest to form and develop as hood-shaped organs, enclosing the inner structures in both wild-type and transgenic spikelets (Figure 3, B and E). Dissection of the lemma and palea exposes the developing lodicules and stamens. Differentiation of stamen primordia into anthers and filaments occurs comparably in wild-type and transgenic spikelets (Figure 3, C, F, and I). Wild-type lodicules initiate as a fleshy cup-shaped structure with a broad basal and a narrow apical end (Figure 3C). Lodicule differentiation in transgenic spikelets deviates from this early stage. While the transgenic lodicule is also cup shaped, it is flattened to a greater extent, thereby losing the typical thick fleshy characteristic seen in wild-type lodicules (Figure 3F). The epidermal cell surface features of these transgenic lodicules are also distinct from those in the wild type (compare Figure 3G with Figure 3H). Further, the apical end of transgenic lodicules continues to grow instead of terminating growth, as seen in comparably staged wild-type lodicules (compare Figure 3I with Figure 3C).

Since rice has two *PI/GLO*-like genes, *OsMADS2* and *OsMADS4*, that share substantial sequence similarity, especially in the MADS box (89% identical), we have examined the specificity of the *OsMADS2* knockdown in our transgenic lines. Northern blot analysis on total RNA from wild-type and pUbi-dsRNAiOsMADS2 transgenic panicles was used to detect endogenous *OsMADS2* transcripts and none were found in transgenic plants (Figure 4A). This suggests a complete transcriptional knockdown of *OsMADS2*. As expected, the 2.3-kb transcript from the transgene (Ds OsMADS2: antisense, linker, and sense transcript) was detected (Figure 4A). This RNA is partially processed into small (~24-nucleotide) RNA molecules (our unpublished data), which likely trigger gene silencing. To determine *OsMADS4* transcript levels in the transgenic panicles, RT-PCR was carried out using primers specific to this gene alone. No change in *OsMADS4* transcript levels was found, a finding replicated in Northern blot analysis also (Figure 4B and data not shown).

Although stamens of eudicot and monocot flowers

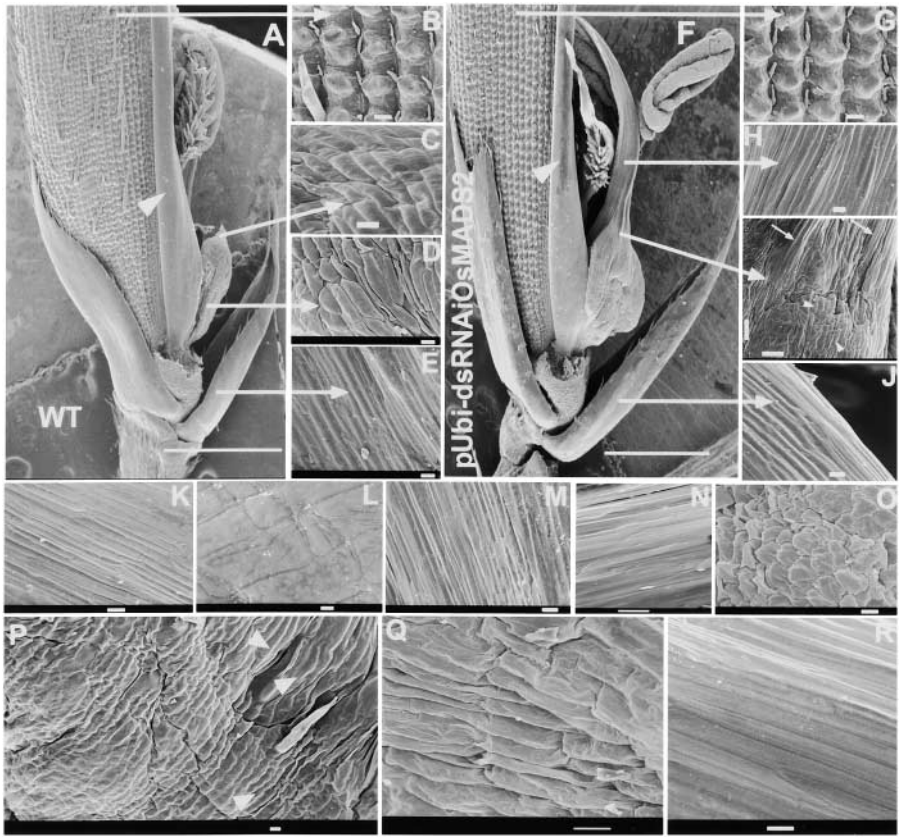

Figure 2.—Comparison of epidermal cell morphologies in sterile whorl organs of a wild-type *vs.* pUbi-dsRNAiOsMADS2 transgenic floret by SEM. (A and F) Partially dissected wild-type and transgenic spikelets, respectively. The lemma has been removed from these florets. Open arrowhead points to the palea marginal tissue. B, C, D, and E show surfaces of wild-type palea, apical/distal lodicule, basal/proximal lodicule parts, and outer glume, respectively. (G) Cell surface of a transgenic palea. (H) Apical portion of a transgenic lodicule with features identical to the wild-type outer glume in E or the marginal tissue of the palea in M. (I) The central portion of a transgenic lodicule showing a mosaic of lodicule is indicated by open arrowheads and glume/palea marginal tissue cell types by open arrows. (J) Abaxial surface of a transgenic outer glume. (K and L) Adaxial surface of a wild-type outer glume and palea, respectively. (M and N) Abaxial and adaxial surfaces of the wild-type palea marginal tissue showing features as in the outer glume. (O) Adaxial surface of a wild-type lodicule. (P) Abaxial surface of the proximal part of a transgenic lodicule. Open arrowheads indicate islands of cells similar to the outer glume or the marginal tissue of palea. (Q) Adaxial surface of the proximal portion of a transgenic lodicule showing atypical elongated cells; compare with O. (R) Adaxial surface of the distal part of the transgenic lodicule with morphological features of outer glume as in K or of the palea marginal tissue as in N. Bars: A and F, 1 mm; B, G, and I, 50 μm; C–E, H, and J–R, 10 μm.

are homologous, the equivalence of lodicules to eudicot petals requires further investigation. Homologous organs can be expected to have a common descent, but monocot and eudicot petals are thought to have arisen independently (Irish 2001). This indicates the likelihood of species-specific mechanisms for petal formation. In grasses, one such mechanism could originate from the duplication of a *PI/GLO* homolog. Here we provide evidence for functional relevance of such duplication in rice. Gene-specific knockdown of one of the rice *PI/GLO*-like genes followed by detailed phenotypic analysis of floral organ differentiation suggests a role only in lodicule development. The homeotic transformation of lodicule cell types to those present in more peripheral organs like the palea or glume, although obvious in these transgenic plants, is not complete. Perhaps B-function activity from *SPW1* (*OsMADS16*) and *OsMADS4* provides some partial lodicule identity. Alternatively, residual *OsMADS2* activity, below our detection limits, persists to specify some lodicule characteristics. Transgenic rice plants expressing an antisense *OsMADS4* cDNA are reported to be defective for both lodicule and stamen development (Kang *et al.* 1998). It is not known whether these cosuppression phenotypes originate from reduced levels of *OsMADS4* alone or from the additional nonspecific cosuppression of *OsMADS2* due to the general effects of antisense RNA on both members of this gene pair. From gross morphology, the defective lodicules of cosuppressed *OsMADS4* transgenic plants were interpreted to be transformed lemma/palea-like organs (Kang *et al.* 1998). However, the study lacked cellular characterization of the transformed organs, and thus it is unclear whether the transformation to lemma/palea cell types was complete or whether any lodicule characteristics persisted. This is a relevant point since transformed second-whorl organs in *spw1*, a mutant in the sole rice *AP3*-like gene, do not have the full complement of all palea cell types. In *spw1* flowers, lodicules acquire characteristics of only the marginal tissue of the palea, which are in fact similar to cell types of the outer glume (Nagasawa *et al.* 2003 and our present study). In this respect, loss of either *SPW1* or *OsMADS2* creates similar transformation of second-whorl organs.

As predicted by the eudicot "ABC" model, rice B-function genes alone are not sufficient for lodicule development and they probably require another class of gene(s). Loss-of-function studies for candidate rice A-function genes are still awaited, as are partners and regulators of *OsMADS2*.

The second phenotype arising from cosuppression of *OsMADS4* is conversion of stamens into carpel-like organs (Kang *et al.* 1998). In contrast, stamens are completely unaffected by loss of *OsMADS2* RNA. Since *Os-*

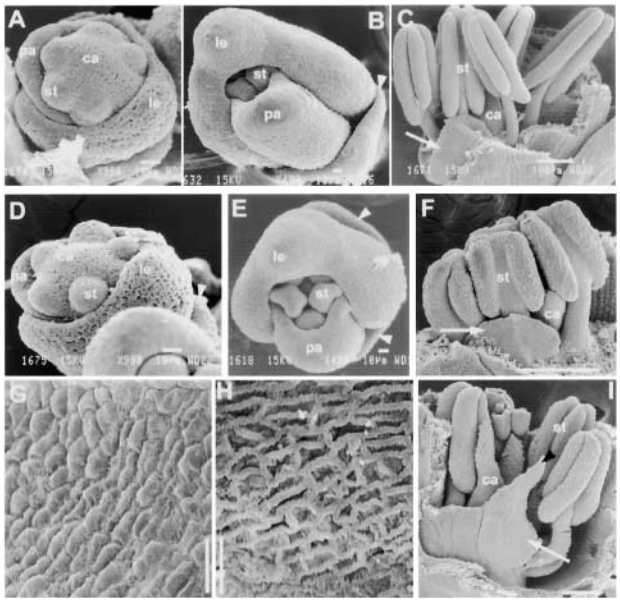

FIGURE 3.—Early developmental stages of floral organogenesis in wild-type and pUbi-dsRNAiOsMADS2 transgenic spikelets. (A) Wild-type floral organ primordia: ca, carpel; st, stamen; primordia are surrounded by developing lemma (le) and palea (pa). (B) A wild-type floret with the lemma (le), and palea (pa) enclosing the inner floral organs. (C) A dissected wild-type floret at a similar developmental stage as that in B. The outer glume, lemma, and palea have been partially dissected to expose the developing lodicule (open arrow) and stamens (st). (D) Floral organ primordia in a pUbi-dsRNAiOsMADS2 transgenic floret. (E) A transgenic floret with nearly complete lemma/palea differentiation. (F) Developing lodicule (open arrow) and stamens (st) in a transgenic floret. The transgenic florets shown in E and F are at a slightly earlier developmental stage than are the wild-type florets in B and C. (G) Epidermal cell surface of a wild-type lodicule at an early developmental stage. (H) Cell surface of a transgenic lodicule at a developmental stage similar to G. (I) A transgenic floret wherein the distal end (solid arrowhead) of the transformed lodicule has continued to grow. This floret is at a developmental stage similar to the wild-type floret in C wherein the distal end of the lodicule has terminated growth. Bars: A, B, D, E, G, and H, 10 μm; C, F, and I, 100 μm.

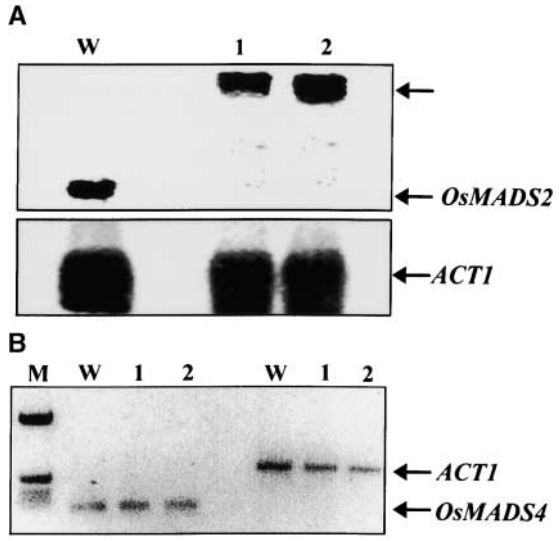

FIGURE 4.—*OsMADS2* and *OsMADS4* transcript levels in wild-type and pUbi-dsRNAiOsMADS2 transgenic panicles. (A) Northern blot analysis of *OsMADS2* transcripts in transgenic (lanes 1 and 2) and wild-type (lane W) plants. Total RNA from these panicles was electrophoresed and probed (top) with a *OsMADS2* gene-specific 450-bp fragment encoding amino acids 181–209 and the 3′ untranslated region (UTR). (Bottom) As a normalization control, the constitutively expressed endogenous rice *ACT1* transcripts were detected. Arrow points to the 2.3-kb transcript from the dsRNAiOSMADS2 transgene. (B) RT-PCR analysis of *OsMADS4* transcripts in wild-type (lane W) and transgenic panicles (lanes 1 and 2). Lane M, molecular weight markers. Total RNA used in A was also utilized for RT-PCR reactions with primers for either a 289-bp fragment of *OsMADS4* encompassing amino acids 167–210 and the 3′ UTR or a 588-bp fragment of the constitutively expressed endogenous rice *ACT1*.

MADS4 expression is unaltered in the latter transgenic plants, it must suffice for normal stamen development. An ancestral PI motif characterizes the C terminus of PI/GLO-like proteins in all flowering plants. Very recent emerging evidence demonstrates that this motif is essential for PI function (LAMB and IRISH 2003). This motif is present in both the rice PI/GLO paralogs. We speculate that the amino acid sequence deviations within the core consensus found in OsMADS2 may underlie its functional divergence.

The only dicot B-function mutant that alters only second-whorl organ (petals) development is *green petal* (*gp*) of petunia. Although this petunia B-function gene is expressed in both second- and third-whorl organs, homeotic conversion occurs only in the second whorl of the *gp* mutant wherein petals are converted to sepals (KUSH *et al.* 1993; VAN DER KROL *et al.* 1993). *GP* has been predicted to function redundantly with another petunia B class gene, *PhBX*, for stamen specification (TSUCHIMOTO *et al.* 2000). Here we provide the evidence that the rice B-function gene *OsMADS2* that is specifically required for second-whorl (lodicule) development is dispensable for specifying the third whorl. Our data also shed light on the importance of *PI/GLO*-like gene duplication for functional diversification and grass floral organ patterning.

We thank the members of the SEM facility of the Department of Metallurgy, Indian Institute of Science. K.P. is a recipient of a Senior Research fellowship from Council for Scientific and Industrial Research, government of India.

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Communicating editor: D. Weigel