

# *leafy hull sterile1* Is a Homeotic Mutation in a Rice MADS Box Gene Affecting Rice Flower Development

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Rice contains several MADS box genes. It has been demonstrated previously that one of these genes, *OsMADS1* (for *Oryza sativa* MADS box gene1), is expressed preferentially in flowers and causes early flowering when ectopically expressed in tobacco plants. In this study, we demonstrated that ectopic expression of *OsMADS1* in rice also results in early flowering. To further investigate the role of *OsMADS1* during rice flower development, we generated transgenic rice plants expressing altered *OsMADS1* genes that contain missense mutations in the MADS domain. There was no visible alteration in the transgenic plants during the vegetative stage. However, transgenic panicles typically exhibited phenotypic alterations, including spikelets consisting of elongated leafy paleae and lemmas that exhibit a feature of open hull, two pairs of leafy palea-like and lemma-like lodicules, a decrease in stamen number, and an increase in the number of carpels. In addition, some spikelets generated an additional floret from the same rachilla. These characteristics are very similar to those of *leafy hull sterile1* (*lhs1*). The map position of *OsMADS1* is closely linked to that of *lhs1* on chromosome 3. Examination of *lhs1* revealed that it contains two missense mutations in the *OsMADS1* MADS domain. A genetic complementation experiment showed that the 11.9-kb genomic DNA fragment containing the wild-type *OsMADS1* gene rescued the mutant phenotypes. In addition, ectopic expression of the *OsMADS1* gene isolated from the *lhs1* line resulted in *lhs1*-conferred phenotypes. These lines of evidence demonstrate that *OsMADS1* is the *lhs1* gene.

## INTRODUCTION

In response to floral induction, the inflorescence meristem becomes committed to flowering. *LEAFY* (*LFY*) and *APE-TALA1* (*AP1*) in Arabidopsis and *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) in Antirrhinum are responsible for promoting the specification of floral meristem identity (reviewed in Ma, 1994). The genes required for specifying the fate of floral organ primordia include *AP1*, *AP2*, *AGAMOUS* (*AG*), *PISTILATA* (*PI*), and *AP3* in Arabidopsis and *SQUA*, *PLENA* (*PLE*), *GLOBOSA* (*GLO*), and *DEFICIENS* (*DEF*) in Antirrhinum (reviewed in Weigel and Meyerowitz, 1994). Excluding *AP2*, these floral homeotic genes encode MADS box proteins that are highly conserved transcription factors in plants, animals, yeast, and fungi and that are regulated by the floral meristem identity gene *LFY* (Parcy et al., 1998; Wagner et al., 1999).

Several other MADS box genes have more subtle functions

associated with floral meristem and floral organ identity. Expression of *AG-LIKE2* (*AGL2*), *AGL4*, and *AGL9* of Arabidopsis begins after the onset of expression of floral meristem identity genes but before the activation of floral organ identity genes (Flanagan and Ma, 1994; Savidge et al., 1995; Mandel and Yanofsky, 1998). *DEFH72* and *DEFH200* of Antirrhinum appear to function in mediating interactions between the meristem and organ identity genes through direct interaction with *PLE* (Davies et al., 1996). *FLORAL BINDING PROTEIN2* (*FBP2*) of petunia and *TOMATO GENE5* (*TM5*) of tomato control organ identity as well as determinacy of the floral meristem (Angenent et al., 1994; Pnueli et al., 1994). Bonhomme et al. (1997) proposed that *SaMADS D* in *Sinapis alba* may act in inflorescence meristem identity and interact with genes specifying floral organ identity. However, the function of many MADS box genes of the *AP1* and *AGL9* group (Purugganan et al., 1995) remains unknown.

The identification of regulatory genes expressed in cereal spikelets has resulted in a greater understanding of the molecular basis of flower development. Two *AG* orthologs of

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maize, *ZAG1* and *ZMM2*, have each evolved separate but partially overlapping activities (Mena et al., 1996). *Silky1*, a maize ortholog of *AP3*, has been cloned by direct transposon tagging (Schmidt and Ambrose, 1998). *INDETERMINATE SPIKELET1*, a member of the *AP2* gene family, is required for determining the spikelet meristem fate and thereby limits the number of floral meristems produced in maize (Chuck et al., 1998).

Several MADS box genes that play important roles in controlling flower development in rice also have been studied (Chung et al., 1994, 1995; Kang et al., 1995, 1997; Greco et al., 1997; Kang and An, 1997; Lopez-Dee et al., 1999; Moon et al., 1999a, 1999b). Using antisense experiments, Kang et al. (1998) previously demonstrated that rice MADS box genes *OsMADS3* (for *Oryza sativa* MADS box gene3) and *OsMADS4* are the putative orthologs of *AG* and *PI*, respectively. Based on its expression pattern, amino acid sequence similarity, and interaction with *OsMADS4* in yeast, *OsMADS16* has been proposed as a homolog of *AP3* (Moon et al., 1999a).

Mutants are useful for the functional analysis of a given gene. When genetic mutants are not readily available, inactivation of a gene function by cosuppression or by using an antisense strategy has been undertaken to elucidate what role the gene plays (Angenent et al., 1993, 1994; Pnueli et al., 1994; Mizukami and Ma, 1995; Kang et al., 1998). Alternatively, for identifying proteins that are parts of a complex, one can generate dominant-negative forms such that the altered protein can inhibit the normal function of the coexisting endogenous one. In *Arabidopsis*, an *AG* protein lacking a C-terminal region inhibits normal *AG* function, generating a phenotype similar to that of *ag1* mutant (Mizukami et al., 1996). Finally, ectopic expression of a gene often provides valuable information regarding its function (Mizukami and Ma, 1992; Jack et al., 1994; Weigel and Nilsson, 1995). In this study, we generated transgenic plants expressing mutant forms of *OsMADS1*, a rice MADS box gene, to get a clue about possible functions of the gene. Through analysis of phenotype of the transgenic plants, we found that inhibition of *OsMADS1* function results in the phenotype similar to that of *leafy hull sterile1* (*lhs1*) (Kinoshita et al., 1976). Moreover, wild-type *OsMADS1* rescued the mutant phenotype, demonstrating that *lhs1* is a homeotic mutation of *OsMADS1*. Finally, by examining the *lhs1* spikelets, we conclude that this MADS box gene plays important roles in determining floral meristem identity and in floral organ development.

## RESULTS

### Expression of the C-Terminal Truncated *OsMADS1* Gene in Rice Plants

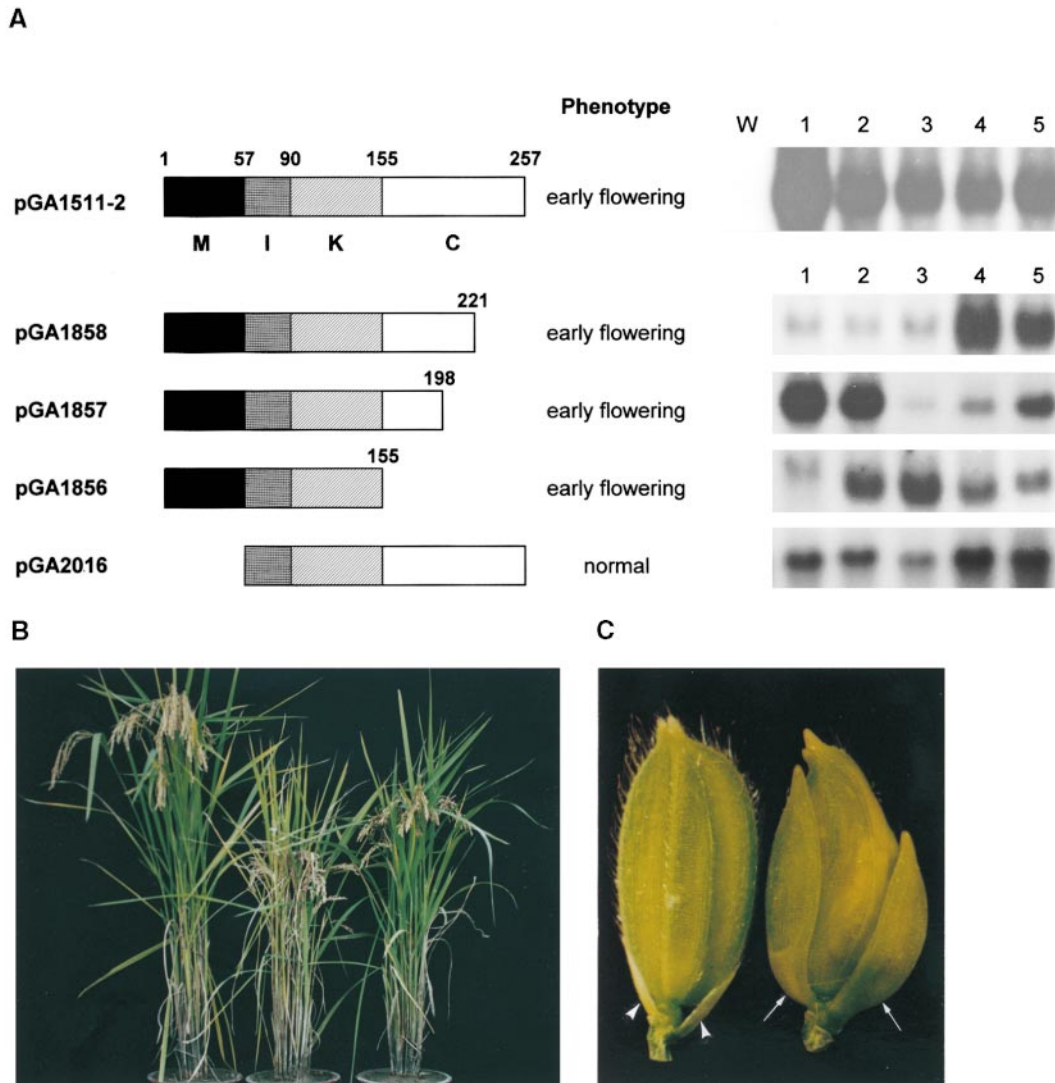
The functional role of *OsMADS1* was investigated by overexpression of the wild or mutant forms of the gene in rice

plants. We generated transgenic rice plants expressing *OsMADS1* constitutively under the control of the rice *act1* (*act1*) promoter (Figure 1A, pGA1511-2). Most of the primary transgenic plants exhibited severe dwarfism, and their panicles were partly embedded in leaf sheaths (Figure 1B, center), compared with the control plants transformed with the binary vector pGA1671 (Figure 1B, left). Transgenic plants flowered 5 to 10 days earlier than did the wild-type controls and exhibited remarkably shorter panicles. RNA gel blot analysis revealed that much more transgene was expressed than wild type (Figure 1A, right). In addition, we observed that two glumes overgrew in transgenic plants that were strongly expressing the transgene, mimicking the palea and lemma (Figure 1C). These results show that overexpression of the *OsMADS1* gene promotes determination of the floral meristem and formation of paleae and lemmas. It had been observed previously that gene expression begins in floral meristems at an early stage of flower development and is strongly expressed in paleae and lemmas at a later stage (Chung et al., 1994).

To understand the function of *OsMADS1*, three mutants (pGA1856, pGA1857, and pGA1858) lacking the C-terminal region and a mutant (pGA2016) lacking the MADS domain were constructed (Figure 1A). The truncated proteins were expressed in rice plants by using the *act1* promoter. Interestingly, transgenic rice plants expressing the C-terminal deletion of the gene had the early-flowering and dwarf phenotypes (Figure 1B, right), although to a lesser extent than the transgenic plants expressing the wild-type *OsMADS1* (Figure 1B, center). There was no obvious alteration of floral organs, except for an occasional elongation of glumes, as was observed for transgenic plants expressing the wild-type *OsMADS1* (Figure 1C). RNA gel blot analyses revealed that all transgenic plants showing early flowering expressed the introduced genes abundantly, indicating that the altered phenotype most likely reflected the expression of *OsMADS1* (Figure 1A). On the other hand, ectopic expression of the *OsMADS1* mutant lacking the MADS domain (pGA2016) showed no phenotypic alteration, probably because of a lack of dimerization and DNA binding. As was previously demonstrated, *AG* genes lacking the MADS domain fail to dimerize and bind DNA (Mizukami et al., 1996). These results indicate that whereas the MADS box region is essential, the C-terminal region is not needed for inducing the early-flowering and dwarf phenotypes.

### Expression of *OsMADS1* Containing Missense Mutations in the MADS Box

Not only is the MADS domain region required for DNA binding, but in some MADS domain proteins the region is also involved in either homo- or heterodimerization (reviewed in Shore and Sharrocks, 1995). Therefore, we postulated that mutations in this region would interfere with the function of MADS genes. To test the hypothesis, seven mutants in the



**Figure 1.** Construction of Truncated *OsMADS1* and Phenotype of Transgenic Plants.

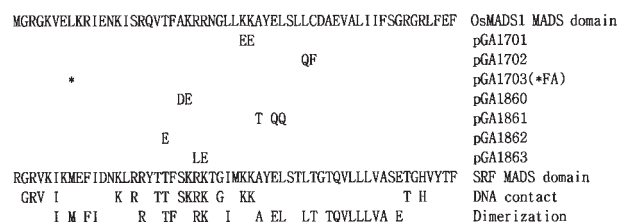
**(A)** Schematic representations of truncated *OsMADS1* proteins, phenotype, and RNA gel blot analyses of the transgenic plants. Shown at left is the *OsMADS1* protein represented by four regions: M, MADS domain; I, I region; K, K domain; C, C-terminal region. The numbers indicate the boundaries of the regions and the positions of the truncations. In the center is the phenotype of the T2 transgenic rice plants that express the construct given at left. Shown at right are RNA gel blot data from the independent transgenic plants, which were derived from transformation of the constructs shown at left. Equal amounts of total RNA loading were examined by using ethidium bromide staining of 25S and 18S rRNAs (data not shown). In pGA1511-2 transgenic lines, line 1 flowered ~10 days earlier than wild-type or control plants carrying the binary vector pGA1671; the other lines flowered ~5 days earlier than controls. In pGA1858, pGA1857, and pGA1856 transgenic lines, lines 1858-4, 1858-5, 1857-1, 1857-2, 1857-5, 1856-2, 1856-3, 1856-4, and 1856-5 flowered ~5 days earlier than controls, and other lines did not show early flowering. Five micrograms of total RNA from prepared leaves was loaded in all other lanes. The C-terminal region of *OsMADS1* was used as a gene-specific probe (Chung et al., 1994), except that RNAs from pGA1856 lines were hybridized with the K region of *OsMADS1*. W, wild-type plants; numbers, independent transgenic lines.

**(B)** Phenotypes of transgenic plants expressing full-length *OsMADS1* or the C-terminal truncated form. Shown at left is a control plant transformed with the binary vector pGA1671; at center is the transgenic plant with pGA1511-2 (line 1511-2-1); at right is the transgenic plant with pGA1856 (line 1856-2).

**(C)** Spikelets of the wild type (left) and transgenic line 1511-2-1 (right). Normal glumes are short and inconspicuous (arrowheads), whereas the transgenic spikelet shows overgrowth of glumes (arrows) that resemble paleae and lemmas. This phenotype was observed in most transgenic plants expressing the full-length or the C-terminal-truncated *OsMADS1*.

MADS box region of *OsMADS1* were generated (Figure 2). In two constructs (pGA1701 and pGA1860), the mutations were in a region corresponding to DNA binding residues of a human MADS box gene, *SRF* (Norman et al., 1988). Three constructs (pGA1702, pGA1703, and pGA1861) carried mutations in amino acid sequences corresponding to the serum response factor (SRF) residues that were involved in dimerization. The remaining two constructs (pGA1862 and pGA1863) were made by introducing missense mutations into amino acid sequences corresponding to the SRF residues involved in both DNA contacting and dimerization. These mutant genes were placed under the control of the *act1* promoter and were introduced into rice plants by means of an *Agrobacterium*-mediated transformation. During the vegetative growth stage, transgenic plants grew normally and were indistinguishable from wild-type plants. There was no significant reduction in plant height or flowering time, indicating that the mutant *OsMADS1* did not behave like the wild-type or the C-terminal truncated forms of the gene.

Rather than the early-flowering and dwarf phenotypes, most of the transgenic plants produced abnormal spikelets that carried elongated papery leafy paleae and lemmas (Figure 3A). Whereas wild-type spikelets are enclosed within the palea and lemma, the mutants had an open hull because of a leafy characteristic of the palea and lemma (Figures 3A and 3B). The inner whorls of wild-type spikelets comprise a pair of lodicules, six stamens, and a carpel (Figure 3D). Abnormal structures observed in the inner whorls of mutant spikelets included two pairs of leafy palea- and lemma-like lodicules, fewer stamens (Figures 3A and 3C), and an occasional additional carpel (data not shown). Some flowers produced an additional palea- and lemma-like leafy structure (Figure 3E). These mutant phenotypes were not observed, however, for plants overexpressing either the wild-type or the C-terminal truncated *OsMADS1* gene product. RNA gel blot analyses with leaves and flowers of transgenic plants



**Figure 2.** MADS Domain Sequences of the Site-Directed *OsMADS1* Mutants.

At left are the amino acids that differ from those of the wild-type MADS domain sequence. The SRF MADS domain region and the amino acid residues involved in DNA contacting or dimerization are shown. The asterisk indicates two amino acid residues generated in the place of Leu<sup>8</sup>. At right are the binary vectors, which consist of the actin *act1* promoter and the mutant *OsMADS1*.

revealed that the transcripts were abundantly expressed in all lines showing the mutant phenotype (Figure 3F). Transgenic plants with the severe mutant phenotype accumulated a high amount of the transgene transcript. A few normal-looking plants weakly expressed the transgene (data not shown). Although we could not distinguish between the transgene transcript and the wild-type transcript in flowers, a marked increase in the amount of transcript in spikelets should reflect high expression of the transgene.

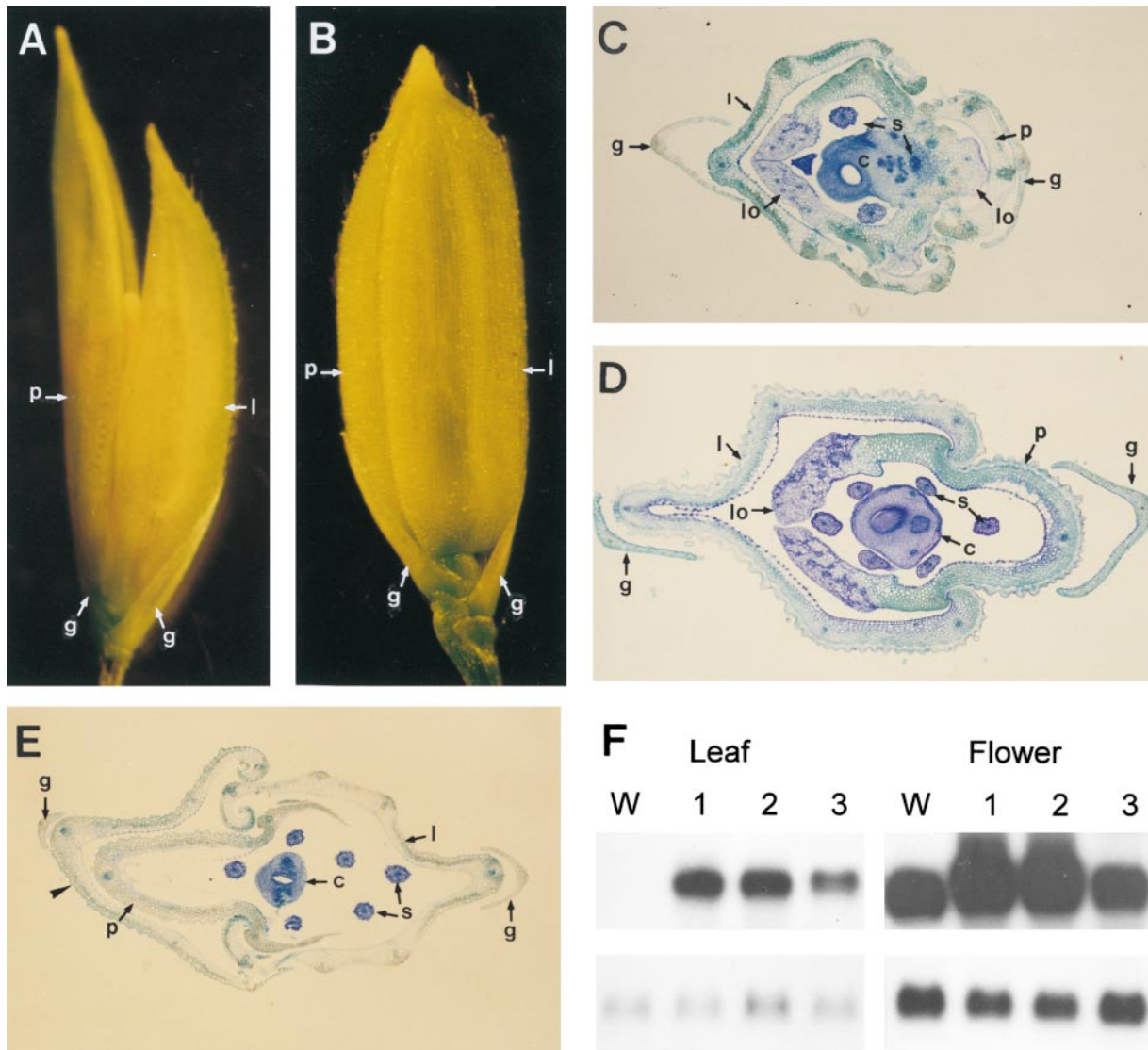
### Chromosomal Mapping of *OsMADS1*

Several mutants in rice display abnormal flower development (Yoshimura et al., 1997). To elucidate whether any of the mutations occur in *OsMADS1*, we determined the location of the gene on a genetic map by using an F11 recombinant inbred population of rice. The result revealed that *OsMADS1* is located between RG100 and RZ313 on chromosome 3 (data not shown). Interestingly, this region also includes *lhs1* (Kinoshita et al., 1976; Khush and Librojo, 1985; Yoshimura et al., 1997), which results in mutant phenotypes that resemble the panicles of the transgenic plants that express *OsMADS1*, which carries missense mutations in the MADS box region.

### Identification of the *OsMADS1* Mutation in *lhs1*

Because the locus of *lhs1* is closely associated with that of *OsMADS1*, we examined whether the *lhs1* mutant line carried any alteration in the *OsMADS1* gene. The coding regions of the *OsMADS1* gene were isolated from the *lhs1* mutant line by using polymerase chain reaction primers located within introns. Sequence analysis of the amplified fragments revealed that the nucleotides C and G at positions 70 and 80, respectively, in the coding region (Chung et al., 1994) were changed to T and A, respectively. Consequently, the arginine of codon 24 and the glycine of codon 27 were replaced with cysteine and aspartic acid, respectively, in the *lhs1* mutation (Figure 4). Alignment of the MADS domains of various MADS box genes from rice and Arabidopsis shows that the amino acids in the region in which the *lhs1* mutation occurred are conserved in all MADS genes, as previously observed (reviewed in Shore and Sharrocks, 1995). Pellegrini et al. (1995) have reported that the Arg<sup>24</sup> is involved in both DNA contacting and dimerization and that the Gly<sup>27</sup> is located at the DNA contacting position. The results presented in Figure 3 show that replacing one to three amino acids in the MADS domain alters the floral organ development, the alterations being quite similar to those for *lhs1*. Therefore, the homeotic alterations in the *lhs1* flowers are likely to have resulted from the mutations in the *OsMADS1* gene.

Point mutations in the MADS domain were also found in homeotic mutants of Arabidopsis and Antirrhinum. These



**Figure 3.** Phenotypes of Transgenic Plants Expressing the *OsMADS1* Mutant.

(A) A spikelet from a plant of transgenic line 1703-1 transformed with pGA1703. The spikelet has a phenotype similar to *lhs1*, showing an open flower with the elongated leafy palea and lemma.

(B) A spikelet of a wild-type plant.

(C) A cross-sectioned spikelet from a plant of line 1703-1. The spikelet consists of normal glumes, abnormal palea and lemma, two pairs of leafy palea- and lemma-like lodicules, four stamens, and a carpel.

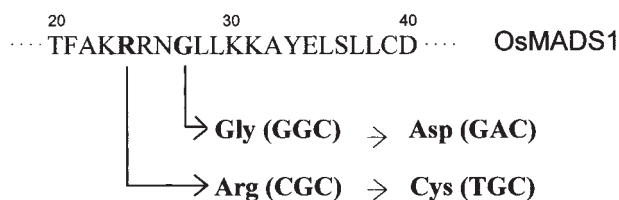
(D) A cross-sectioned wild-type spikelet. The spikelet has glumes, palea and lemma, a pair of lodicules, six stamens, and a carpel.

(E) A spikelet from the line 1703-3. The spikelet has an additional palea- and lemma-like structure (arrowhead). Original palea and lemma developed abnormally. Other organs—lodicules, stamens, and a carpel—are almost identical to the wild type.

(F) RNA gel blot analysis of the *OsMADS1* transcript in the leaves and flowers of the 1703 transgenic lines. Five micrograms of total RNA from leaves (left) and 10  $\mu$ g from flowers (right) were used for RNA gel blot analysis. At top, the amount of *OsMADS1* transcript expressed was measured using the gene-specific probe for *OsMADS1*. Because of the similar sizes of the endogenous and transgenic *OsMADS1* transcripts, the signals shown are the sum of the two. Lanes W, wild type; lanes 1, line 1703-1; lanes 2, line 1703-2; lanes 3, line 1703-3. At bottom are the controls. The same filters were washed and rehybridized with the rice  $\alpha$ -tubulin gene *OsTubA1* (Jeon et al., 2000).

c, carpel; g, normal glume; l, abnormal lemma; lo, leafy palea- and lemma-like lodicules; p, abnormal palea; s, stamen.





**Figure 4.** Amino Acid Changes Corresponding to Nucleotide Changes in the *lhs1* Allele.

The amino acid sequence is in the MADS box domain. Boldface letters indicate the changes in the *lhs1* allele.

include *ap1-2*, *cauliflower-2 (cal-2)*, *cal-3*, and *pi-3* in Arabidopsis and *defa-nicotianoides* in Antirrhinum (Mandel et al., 1992; Schwarz-Sommer et al., 1992; Goto and Meyerowitz, 1994; Kempin et al., 1995). In particular, the mutant *ap1-2*, *cal-3*, and *defa-nicotianoides* alleles result from a missense mutation of Gly<sup>27</sup> to Asp, the same alteration as in *lhs1* (Mandel et al., 1992; Schwarz-Sommer et al., 1992; Kempin et al., 1995). These results support the importance of the 27th amino acid residue for MADS box gene function.

#### Genetic Complementation of *lhs1* by *OsMADS1*

To investigate whether the phenotype of the *lhs* mutant was indeed attributable to the alterations of *OsMADS1*, we performed a complementation experiment, using the wild-type *OsMADS1* clone isolated from a genomic library. Restriction mapping and DNA gel blot analysis of the genomic clone showed that the 11,851-bp EcoRI fragment carried the entire *OsMADS1* gene (Figure 5A, top). The fragment consisted of a 1917-bp 5' upstream sequence, eight exons, seven introns, and a 1484-bp 3' region (Figure 5A, center). The first intron was 5524 bp long and located in codon 62. The other introns were much shorter, ranging between 90 and 771 bp. All introns contained the consensus GT and AG sequences at the 5' and 3' ends, respectively. Neither the 5' upstream region, the 3' downstream region, nor the introns contained any open reading frames of notable length, thus indicating that the 11,851-bp EcoRI fragment carries only the *OsMADS1* gene.

The genomic DNA fragment that included the entire wild-type *OsMADS1* gene along with the large intron was cloned into the binary vector pGA1182 (Figure 5A, bottom). A large intron is often present after the MADS box region, such as in *AG* of Arabidopsis and *PLE* and *FARINELLI* of Antirrhinum. Insertion of T-DNA or a transposon in the intron of these genes resulted in loss of gene function (Yanofsky et al., 1990; Bradley et al., 1993; Davies et al., 1999). The binary vector pGA2122, carrying the entire *OsMADS1* gene, was introduced into the Agrobacterium strain LBA4404 and used for transformation of the *lhs1* plants. Four of nine independently transformed plants generated spikelets that were

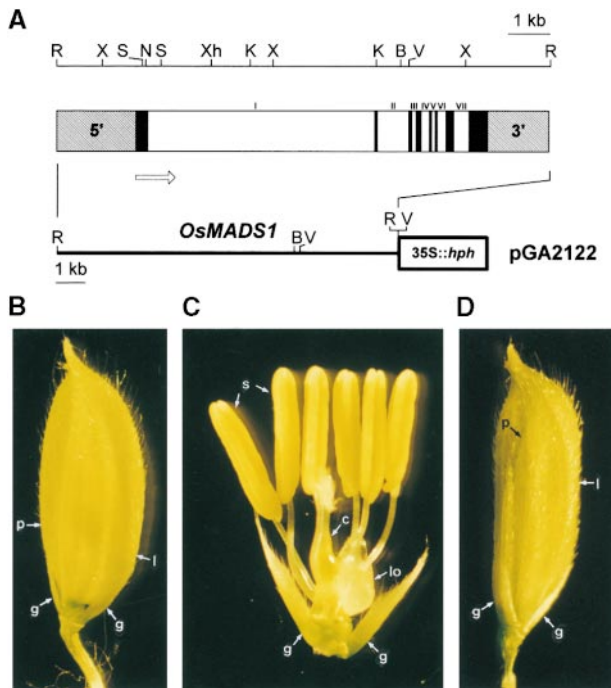
completely recovered from the mutant phenotype (Figures 5B and 5C). Another four lines showed a partial recovery (Figure 5D). Control transgenic plants transformed with the vector pGA1182 did not show any complementation of the *lhs1*-conferred phenotype (data not shown). Genomic DNA gel blot analysis revealed that all four of the transgenic lines that completely rescued the mutant phenotype contained both the wild-type *OsMADS1* gene and the *lhs1* allele (data not shown). These results strongly support the hypothesis that the phenotype of the *lhs1* mutant is caused by mutations in the *OsMADS1* gene.

#### Spikelets of the *lhs1* Mutant

To examine the functional role of *OsMADS1* during flower development, we analyzed panicle development in the *lhs1* mutant (Figure 6). We observed that spikelets of the *lhs1* mutant contained leafy paleae and lemmas that resulted in open flowers (Figures 6A and 6B). A pair of lodicules became leafy, resembling the palea and lemma. The number of stamens was reduced to four, on average (Figures 6C and 6H), although occasionally an additional carpel developed (Figure 6I). In some *lhs1* spikelets, a new abnormal flower was formed in a whorl of stamens from the same rachilla (Figures 6D and 6E) or from a stigma of a carpel (Figure 6F), indicating that the *lhs1* spikelets had incomplete floral meristem determination. Flowers with eight stamens were rarely observed, possibly because of the generation of a new flower in a spikelet (Figure 6J). In all spikelets, glumes developed normally (Figures 6A to 6C), and most anthers in *lhs1* flowers produced normal pollen (Figure 6J).

We compared the morphology of wild-type flowers with that of *lhs1* flowers at various developmental stages, using scanning electron microscopy (Figure 7). In the floral primordium of a wild-type flower, the glumes, palea, and lemma developed first (Figure 7A), after which the whorls of lodicules and stamens became apparent (Figure 7B). While the stamen primordia were continuing to grow, the remaining tissue of the central meristem elongated and gave rise to the carpel primordium (Figure 7C). Anther and stigma precursors and lodicules then became distinct (Figure 7D). At a late stage of flower development, anther locules and filaments differentiated (Figure 7E). At this point, unlike Arabidopsis petals, the second whorl of the lodicules did not elongate (Smyth et al., 1990). The stigmatic papillae of a carpel were then developed (Figure 7F). Figure 7G shows a mature flower of the wild-type spikelet before anthesis. Long trichomes appeared more abundantly on veins of palea and lemma.

The *lhs1* spikelets were distinguishable from those of the wild type when floral primordia began to develop (Figure 7H). At this stage, a new palea and lemma structure was seen before the formation of stamen and carpel primordia. The palea and lemma began to overdevelop at an early stage of flower development (cf. Figures 7H to 7J with 7A to



**Figure 5.** Structure of the *OsMADS1* Genomic Clone, the Vector Used for Genetic Complementation, and Phenotypes of the Transgenic Plants.

(A) A restriction map of the *OsMADS1* genomic clone is shown at top. B, BamHI; K, KpnI; N, NotI; R, EcoRI; S, SacI; V, EcoRV; X, XbaI; Xh, XhoI. At center is the genomic structure of *OsMADS1*. The 11,851-bp EcoRI fragment contains the entire *OsMADS1* gene, which consists of seven introns (white bars) and eight exons (black bars) (GenBank accession number AF204063). The length of each intron is, in order from left, 5524, 771, 120, 207, 90, 218, and 378 bp. The numbers on top of the DNA fragment indicate the introns. The promoter and terminator regions are shown in the diagonally striped rectangles. The open arrow indicates the direction of transcription. At bottom is the binary vector pGA2122 containing the *OsMADS1* genomic clone. The vector also harbors the hygromycin phosphotransferase (*hph*) gene under the control of the 35S promoter for selection of transgenic plants.

(B) A spikelet from a transgenic plant from line 2122-4. The structure of palea and lemma in plant 2122-4 is indistinguishable from that of a wild-type spikelet.

(C) A dissected spikelet from a plant of line 2122-4 showing inner floral organs. The spikelet consists of a pair of lodicules, six stamens, and a carpel, which are identical to those of the wild type.

(D) A spikelet from a transgenic plant from line 2122-6. The palea is partially recovered.

c, carpel; g, normal glume; l, abnormal lemma; lo, leafy palea- and lemma-like lodicules; p, abnormal palea; s, stamen.

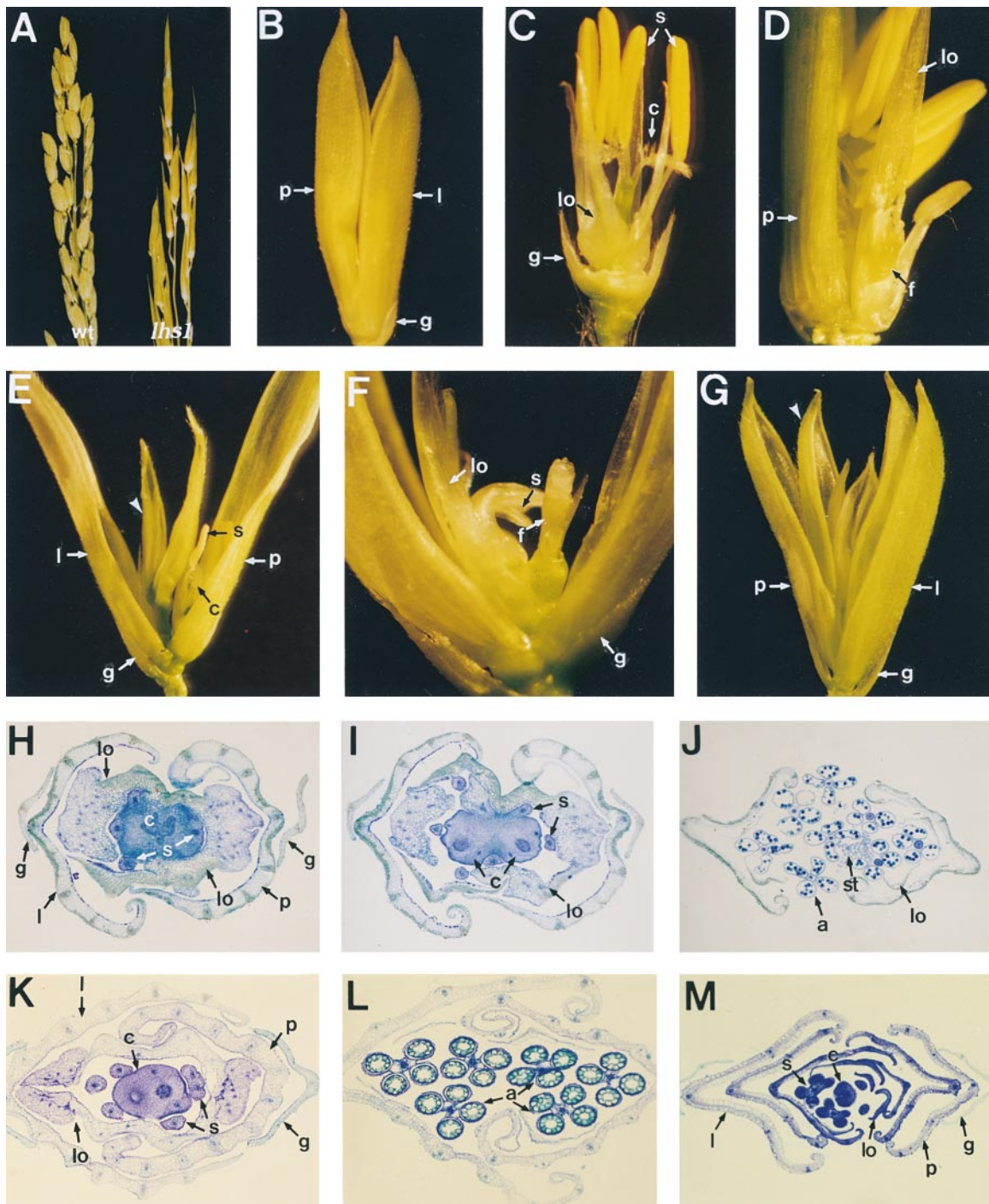
7C). Stamen and carpel primordia were formed irregularly (Figures 7I and 7J), with the number of stamens and carpels varying from two to eight and one or two, respectively, in *lhs1* spikelets (Figures 7K to 7M). At the later stage, when stamens began to elongate, the *lhs1* spikelets formed open hulls (Figure 7K), and the first whorl primordia eventually became the leaflike structures (Figure 7N). As anthers and filaments differentiated, lodicules were developed to leafy structures (Figure 7L). In some *lhs1* spikelets, a new flower appeared between a stamen and a palea and lemma (Figure 7M). Because the floral primordium initiated later, the new flower appeared to be younger than the primary flower. In leafy hulls of mature spikelets, trichomes were underdeveloped (Figure 7N) compared with those in the wild type (Figures 7G). Some flowers carried several leafy paleae and lemmas within a spikelet (Figure 7O).

To examine whether overexpression of *lhs1* acted as a dominant-negative form, the *OsMADS1* cDNA clone from the *lhs1* mutant was placed under control of the maize ubiquitin promoter that showed strong activity in monocots (reviewed in McElroy and Brettell, 1994), and the construct was introduced to a wild-type rice plant. Six of the seven transgenic lines showed phenotypes that were similar to that of *lhs1*. Of these, four lines showed a mild phenotype of *lhs1* (Figures 6K and 6L). The structure of the spikelets in the four lines was similar to those shown in Figures 6B, 6C, and 6H. In two lines, 2145-9 and 2145-11, some of the flowers developed more than two floral primordia within a spikelet (Figure 7P). Consequently, the number of leafy paleae and lemmas was increased, and new leafy palea and lemma formed successively in the spikelet (Figures 6G and 6M), indicating that the flowers have partial inflorescence characteristics.

Kinoshita et al. (1976) previously reported that *lhs1* in the winter season produces spikelets composed of many leafy paleae and lemmas without a carpel and stamens. It will be interesting to determine whether the expression level of *lhs1* or of a dimerization partner that interacts with LHS1 changes during winter. These results suggest that mutations in the MADS domain of *OsMADS1* act as a dominant-negative form when they are strongly expressed. We did not observe the phenotypes of dwarfism and early flowering that appeared when wild-type *OsMADS1* was expressed constitutively in rice plants, which indicates that the *lhs1* allele acts specifically in flower tissues in a dominant-negative fashion.

**DISCUSSION**

In this study we have elucidated a role of the *OsMADS1* gene by expressing mutant forms of the gene. Missense mutations in the MADS domain of *OsMADS1* caused abnormal spikelets similar to those caused by *lhs1* in transgenic rice plants. This phenotype was not observed for transgenic plants expressing either the wild-type *OsMADS1* or the



**Figure 6.** Spikelets from *lhs1* and Transgenic Plants.

(A) Panicles from wild-type (left) and *lhs1* (right) plants.

(B) A spikelet from the *lhs1* mutant. The palea and lemma are overdeveloped in comparison with those of the wild type (see Figure 3B). The spikelet is open because of abnormal growth of leafy palea and lemma.

(C) A dissected *lhs1* spikelet in which a palea and lemma were ripped off.

(D) An *lhs1* spikelet generating a new floret.

(E) An *lhs1* spikelet generating a new flower (arrowhead) consisting of leafy palea and lemma.



forms that lacked the MADS box or the C terminus. This result, together with the fact that several mutations in the MADS domain all resulted in similar phenotypes, suggests that the abnormal spikelet development is caused by formation of a defective complex between mutant *OsMADS1* and wild-type *OsMADS1* or other MADS proteins. This defective complex may not properly recognize its target sequence, thereby resulting in a lack of proper function. Considering that these *OsMADS1* mutants did not induce early flowering and dwarfism, it is also possible that the complex binds to a false target sequence or to another protein that is not a normal partner in wild-type spikelets.

The *lhs1* mutation has been reported to be a recessive allele (Khush and Librojo, 1985). However, the transgenic plants expressing the mutated *OsMADS1* gene displayed phenotypes of abnormal spikelet in the primary transgenic lines, indicating that the mutant *OsMADS1* functions as a dominant allele. This discrepancy might reflect a difference in the expression level of the mutant protein. In *LHS1/lhs1* plants, the amount of the mutant *OsMADS1* protein produced is expected to be equal to that of the wild-type *OsMADS1* protein. This amount of mutant protein was probably insufficient to have had negative effects on other proteins. However, in transgenic plants displaying the mutant phenotype, the amount of mutant protein must have been much higher than that of the wild-type protein because a strong promoter was used for expression of the transgene.

The mutant phenotypes do not appear to have been caused by cosuppression of the endogenous *OsMADS1* gene. RNA gel blot analysis demonstrated that the amount of total *OsMADS1* mRNA in flowers of transgenic plants was more than that in wild-type flowers. *OsMADS1* mRNA was also detected in high abundance in the leaves of transgenic plants. In addition, the altered phenotype was observed at a high frequency in the transgenic lines. Ordinarily, the frequency of cosuppression is not this high (Angenent et al., 1993, 1994). Therefore, the missense mutant forms in the MADS domain of *OsMADS1* apparently behave as a dominant-negative form. Krizek et al. (1999) have reported that overexpression of AP3 or AG lacking the N-terminal region of the MADS domain caused the mutant phenotype, although

the frequency of the mutant phenotype was not as great as the frequency at which the *lhs1* phenotype occurred, as seen in the expression of the missense mutant forms. Probably a lack of DNA binding ability in the abnormal dimers caused a defect of the gene function.

Transgenic plants overexpressing the C-terminal deletion mutants of *OsMADS1* had the weak early-flowering and dwarf phenotypes, which are typical in transgenic plants expressing the wild-type *OsMADS1*. Therefore, the C-terminal region does not appear to be required for inducing early flowering. Several pieces of evidence demonstrate the importance of the C terminus of plant MADS box genes. Three *ap1* alleles and a *ca1* allele have been reported to result from mutations in the C-terminal region (Kempin et al., 1995; Egea-Cortines et al., 1999). Recent studies with the yeast system have demonstrated that three MADS box proteins—SQUA, DEF, and GLO—form ternary complexes by way of their C termini (Egea-Cortines et al., 1999). We have also demonstrated with yeast and mammalian cells that the C terminus of *OsMADS1* contains a transcription activation motif (J. Lim, Y.-H. Moon, G. An, and S.K. Jang, manuscript submitted for publication). The results suggest that the C terminus of a MADS box protein is important for specific functions of these proteins.

Because deletion of the C-terminal region from *OsMADS1* did not interfere with its role for inducing early flowering, the early-flowering phenotype resulting from ectopic expression of *OsMADS1* might show a negative effect of the gene product. Perhaps *OsMADS1* negatively regulates other gene products that are required for the duration of vegetative growth in the shoot apex. If that is the case, then the *OsMADS1* C terminus is not needed for regulation. In the yeast two-hybrid system, *OsMADS1* that lacks a C terminus forms a heterodimer with other MADS domain proteins, namely, *OsMADS6*, *OsMADS14*, *OsMADS15*, and *OsMADS17* (Moon et al., 1999b; S. Jang and G. An, unpublished data). Therefore, ectopic *OsMADS1* might interact with a MADS domain protein that acts as a floral repressor, such as FLOWERING LOCUS C (Michaels and Amasino, 1999), thereby inducing early flowering. It is also possible that the formation of a complex by several proteins is needed to

**Figure 6.** (continued).

(F) An *lhs1* spikelet generating a new flower in sequence.

(G) A transgenic spikelet from transgenic line 2145-9 generating multiple paleae and lemmas (arrowhead).

(H) A cross-section of an *lhs1* spikelet. The spikelet consists of normal glumes, abnormal leafy palea and lemma, two pairs of leafy palea- and lemma-like lodicules, four stamens, and a carpel.

(I) A cross-section of an *lhs1* spikelet. The spikelet consists of leafy palea and lemma, abnormal lodicules, six stamens, and two carpels.

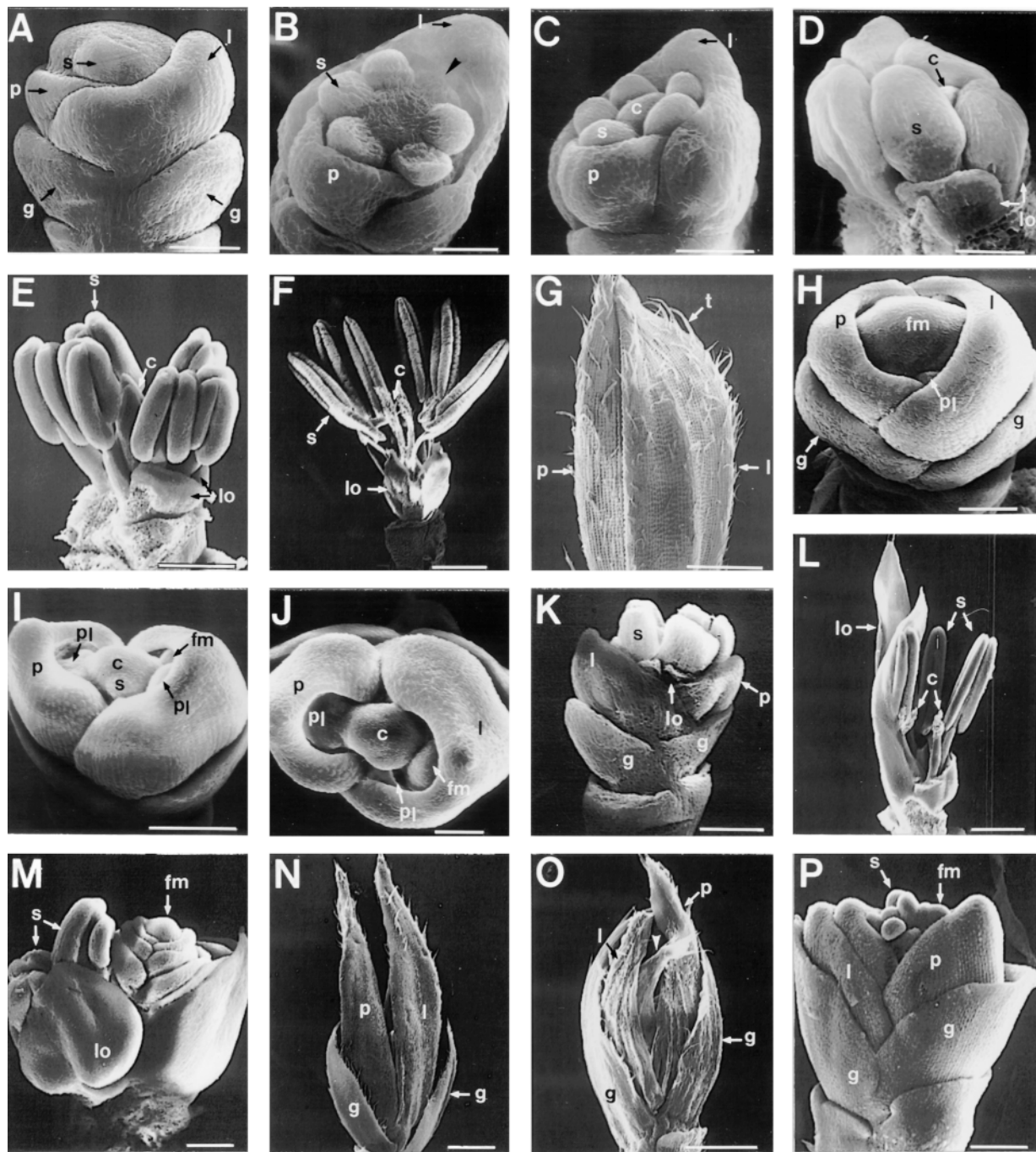
(J) A cross-section of an *lhs1* spikelet. The spikelet consists of two pairs of lodicules, eight stamens, and two carpels.

(K) A cross-section of a spikelet from a plant of transgenic line 2145-8.

(L) A cross-section of anthers of a spikelet from a plant of line 2145-8.

(M) A cross-section of a spikelet from a plant of line 2145-9, showing multiple paleae and lemmas.

a, anther; c, carpel; f, flower; g, glume; l, lemma; lo, lodicule; p, palea; s, stamen; st, stigma.



**Figure 7.** Scanning Electron Microscopy of Wild-Type, *lhs1*, and Transgenic Spikelets.

Scanning electron microscopy of spikelets of wild-type [(A) to (G)], *lhs* [(H) to (O)], and transgenic line 2145-9 (P) rice.

- (A) In outer whorls, glumes and palea and lemma primordia are formed. The central floral meristem starts initiating stamen primordia. Bar = 75  $\mu$ m.  
 (B) Six developing stamens are visible. One stamen primordium is developing late (arrowhead). Removal of the lemma reveals that lodicule primordia form at this stage (data not shown). Bar = 50  $\mu$ m.  
 (C) The carpel primordium is apparent. Bar = 100  $\mu$ m.  
 (D) A dissected spikelet in which palea and lemma were ripped off. A pair of lodicules is formed between stamens and a lemma. Bar = 50  $\mu$ m.  
 (E) In stamens, filaments and anthers are apparently differentiated. Bar = 200  $\mu$ m.  
 (F) The mature spikelet in which palea and lemma were ripped off. Bar = 0.5 mm.  
 (G) Dissected spikelet showing the tassel (t). Bar = 100  $\mu$ m.  
 (H) Dissected spikelet showing the floral meristem (fm) and palea (p). Bar = 50  $\mu$ m.  
 (I) Dissected spikelet showing the palea (p), lodicule (lo), and floral meristem (fm). Bar = 50  $\mu$ m.  
 (J) Dissected spikelet showing the palea (p), lodicule (lo), and floral meristem (fm). Bar = 50  $\mu$ m.  
 (K) Dissected spikelet showing the palea (p), lodicule (lo), and glume (g). Bar = 50  $\mu$ m.  
 (L) Dissected spikelet showing the lodicule (lo), stamen (s), and carpel (c). Bar = 50  $\mu$ m.  
 (M) Dissected spikelet showing the floral meristem (fm), stamen (s), and lodicule (lo). Bar = 50  $\mu$ m.  
 (N) Dissected spikelet showing the palea (p), lodicule (lo), and glume (g). Bar = 50  $\mu$ m.  
 (O) Dissected spikelet showing the palea (p), lodicule (lo), and glume (g). Bar = 50  $\mu$ m.  
 (P) Dissected spikelet showing the stamen (s), floral meristem (fm), palea (p), lodicule (lo), and glume (g). Bar = 50  $\mu$ m.

suppress vegetative growth and induce reproductive growth. Mutants lacking the C terminus of *OsMADS1* would be invaluable in unveiling the role of the C terminus of the gene.

We have demonstrated, on the basis of several lines of evidence, that *lhs1* is the result of mutations in *OsMADS1*. First, the location of *lhs* is mapped near *OsMADS1* on chromosome 3. Second, the phenotype of the *lhs1* mutant is almost identical to those of transgenic plants expressing *OsMADS1* with missense mutations in the MADS box region. Third, the *lhs1* mutant allele carries mutations in the MADS box region of *OsMADS1*. Fourth, introduction of the wild-type *OsMADS1* gene rescues the phenotype of the *lhs* mutant. Fifth, overexpression of the *lhs1* allele in wild-type rice plants induces the phenotype of the *lhs1* mutant.

*lhs1* is a single recessive gene and has been characterized by leaflike transformation of paleae and lemmas (Khush and Librojo, 1985). In the weak phenotype of the *lhs1* mutant, the spikelet consists of leafy palea and lemma, two pairs of palea- and lemma-like structures, fewer stamens, and more carpels. In plants with the strong phenotype, the *lhs1* mutation results in generation of new flowers within the spikelet. Scanning electron microscopy shows that floral meristems of *lhs1* spikelets are irregularly differentiated into floral organs or a new flower. A normal pattern of lodicules, stamens, and carpel development is seldom present in *lhs1* spikelets. This suggests that *OsMADS1* plays a pivotal role in floral meristem determination during the early development of rice flowers. Morphological changes in paleae and lemmas might also result from defects of the *OsMADS1* function at later stages, because the gene is expressed abundantly in mature paleae and lemmas (Chung et al., 1994). Apparently, *OsMADS1* also affects the development of lodicules. The abnormal lodicules in *lhs1* flowers might be the result of downregulation of other MADS box genes that are associated with lodicules. We also do not exclude the possibility that abnormal lodicules may be the product of the

incomplete paleae and lemmas that replaced lodicules in the second whorl.

As previously stated, *OsMADS1* interacts with other MADS domain proteins of the AP1/AGL9 group in a yeast two-hybrid system. Therefore, the phenotype of the *lhs1* mutant might result from formation of nonfunctional dimers or multimers between LHS1 and other MADS domain proteins that are essential for flower development. Khush and Librojo (1985) reported that *overdeveloped palea* and *naked seed rice* are allelic to *lhs1*. Characterization of these mutants would be useful in further understanding the function of *OsMADS1*.

On the basis of sequence homology, *OsMADS1* can be classified in the AP1/AGL9 group (Purugganan et al., 1995). Inactivation of the tomato *TM5* and petunia *FBP2* resulted in a defect of the inner three whorls and development of additional whorls of organs or new flowers in the center of flowers (Angenent et al., 1994; Pnueli et al., 1994). The results suggest that *TM5* and *FBP2* control organ identity as well as determinacy of the floral meristem. In situ hybridization experiments with *AGL9* suggest that the gene may function early in flower development to mediate between expression of floral meristem identity genes and activation of organ identity genes; later, it may control the development of petals, stamens, and carpels (Mandel and Yanofsky, 1998). The present study suggests that *OsMADS1* shares some similarity with *TM5* and *FBP2* with respect to determination of the floral meristem.

Unlike the dicot MADS box genes, *OsMADS1* appears to play an additional role during the development of the palea and lemma at the late stage of flower development. Infrequently, more complex flowers evolved, probably because of an early function of *OsMADS1* in the formation of the floral meristem. We often observed a new flower replacing the whorl of stamens, mimicking the phenotype of the Arabidopsis *ap1*. However, it is premature to conclude that *OsMADS1* is the functional AP1 homolog of rice. The

Figure 7. (continued).

(G) The palea and lemma of a mature spikelet. Bar = 1 mm.

(H) Glume and palea and lemma primordia are formed in outer whorls of a *lhs* spikelet. In the inner whorls, a new palea- and lemma-like structure is visible. Bar = 50  $\mu$ m.

(I) Palea- and lemma-like structures and a floral meristem are formed in the inner whorls of the *lhs1* spikelet. The central meristem is differentiated to stamens and a carpel. Bar = 100  $\mu$ m.

(J) Top view of (I). Bar = 50  $\mu$ m.

(K) The *lhs* spikelet carries five stamens. Lodicules are formed at the base of the stamens. Bar = 100  $\mu$ m.

(L) The spikelet consists of leafy lodicules, three stamens, and two carpels. Palea, lemma, and right lodicule were removed. Bar = 0.5 mm.

(M) The spikelet consists of leafy lodicules, two stamens, and a new flower. Palea and lemma were removed to reveal inner organs. Bar = 100  $\mu$ m.

(N) A mature spikelet. Because of the leafy characteristic of the palea and lemma, the spikelet is open. Bar = 0.5 mm.

(O) The spikelet exhibits successive formation of paleae and lemmas (arrowhead). Bar = 0.5 mm.

(P) Early development of the spikelet from a plant of transgenic line 2145-9. The young spikelet consisted of successive leafy paleae and lemmas and floral primordia. Bar = 100  $\mu$ m.

c, carpel; fm, floral primordium; g, glume; l, lemma; lo, lodicule; p, palea; pl, palea- and lemma-like structure; s, stamen; t, trichome.

phenotype of the *lhs1* mutant includes a decrease in the number of stamens, which has not been reported for *ap1*. We have isolated two rice MADS box genes, *OsMADS14* and *OsMADS15*, the sequences of which are most homologous to *ZAP1*, an *AP1* homolog in maize (Moon et al., 1999b; J. Lim, Y.-H. Moon, G. An, and S.K. Jang, manuscript submitted for publication). Recently, Cacharrón et al. (1999) reported two MADS box genes from maize, *ZMM8* and *ZMM14*, that probably are orthologous to *OsMADS1*. Expression patterns of both maize genes suggest that they may be involved in determining the spikelet meristem and in distinguishing the upper from the lower floret in the maize spikelet. Theissen et al. (1996) grouped *OsMADS1* with two MADS box genes of maize, *ZMM3* and *ZMM8*, on the basis of sequence similarity. Several MADS box genes in rice can be classified in the *AP1* group. Studying these MADS box genes will help us to understand the regulatory mechanisms involved in spikelet development in monocot plants.

## METHODS

### Plant Materials

*leafy hull sterile1 (lhs1)* was recovered by Toshiro Kinoshita (Hokkaido University, Japan) and was made available by H.J. Koh (Seoul National University, Korea). A japonica cultivar, Dongjin, was used for rice transformation.

### Mutagenesis and Vector Constructions

The C-terminal deletion constructs of *Oryza sativa MADS box gene1 (OsMADS1)* were produced by polymerase chain reaction with the *OsMADS1* cDNA, which was cloned in pBluescript SK- (Stratagene, La Jolla, CA) as a template. T3 was used as the forward primer. The reverse primers, which were used for generation of C-terminal-deleted mutants, are as follows: 5'-TATTCCTCGAGGCTGTTGCTACTTGCTTTCAG-3' (pGA1856); 5'-TATTCCTCGAGGATGAGGCTAATCAGCAAGAAC-3' (pGA1857); and 5'-TATTCCTCGAGG-GTGATGTTACCCAATCTGCAGGG-3' (pGA1858).

The bases complementary to the stop codon are underlined. The MADS box deletion construct pGA2016 was generated with the T7 primer and 5'-GCTCTAGACCATGTCCAGCTCATCATG-3'; here, the underlined bases encode a new translation initiation codon. Missense mutations were generated by site-directed mutagenesis. Single-stranded DNA was isolated from an *Escherichia coli* RZ1032 harboring the *OsMADS1* MADS box region, which was subcloned into pBluescript SK-. Mutagenesis was performed according to the method of Kunkel et al. (1991). The primers for mutagenesis are as follows: 5'-GAACGGCCTGCTCGAGGAGGCCTACGAGCT-3' (pGA1701); 5'-TACGAGCTCTCCCTCCAGTTCGACGCCGAGGTCG-3' (pGA1702); 5'-GGAATTCGCCAAGCGGATCGAG-3' (pGA1703); 5'-ACGTTCCGACGAGCGCAGGAAC-3' (pGA1860); 5'-AAGACCTAC-CAGCAGTCCCTC-3' (pGA1861); 5'-TCAGCCGGCAGGTGGAATTCGCCAAGCGCAG-3' (pGA1862); and 5'-GCCAAGCTCGAG-AACGGC-3' (pGA1863). The bases corresponding to the mutated amino acid residues are underlined. All amplified fragments were se-

quenced to verify that the desired mutations had been produced and that there were no other changes in the remaining MADS box region.

The mutated genes were cloned into XbaI and XhoI sites between the actin *act1* promoter (McElroy et al., 1990) and the T7 terminator of the binary vector pGA1671. The control plasmid pGA1511-2 was constructed by insertion of the wild-type *OsMADS1* cDNA into pGA1671.

The *lhs1* cDNA was constructed by replacing the NaeI-NsiI fragment of the wild-type *OsMADS1* cDNA (Chung et al., 1994) with the same fragment of the *lhs1* allele. The constructed cDNA, which encoded the entire *lhs1*-coding region, was placed under the control of the maize ubiquitin promoter and the nopaline synthase terminator by using pGA1611 (Kang et al., 1998). The chimeric molecule was named pGA2145.

### Isolation of the *OsMADS1* Genomic Clone and Genetic Complementation of *lhs1*

A rice genomic library constructed in the  $\lambda$  DASH vector with IR36 DNA (kindly provided by S. Kay, Scripps Institute, La Jolla, CA) was used for isolation of a genomic clone, as described by Sambrook et al. (1989). Phage DNA was prepared by the method of Chisholm (1989). Subcloning and DNA sequencing were performed as described previously (Sambrook et al., 1989). The pGA2122 plasmid was constructed by cloning of the EcoRI genomic fragment containing the entire *OsMADS1* gene into the binary vector pGA1182, which contained the hygromycin phosphotransferase gene as a selection marker.

### Production and Growth of Transgenic Rice Plants

Rice transformation was performed by the *Agrobacterium*-mediated cocultivation methods previously described (Jeon et al., 1999). All transgenic rice plants were generated on 40 mg/L hygromycin B-containing medium. The regenerated plants were grown in a greenhouse kept typically at 30°C during the day and 20°C at night. The light/dark cycle in the greenhouse was 14/10 hr.

### DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from mature leaves at the heading stage, as described previously (Dellaporta et al., 1983). Five micrograms of genomic DNA was digested with EcoRV and BamHI, separated on a 0.7% agarose gel, blotted onto a nylon membrane, and hybridized with a <sup>32</sup>P-labeled probe. Total RNAs were isolated from leaves and flowers at the heading stage by the RNA isolation kit (Tri Reagent; MRC Inc., Cincinnati, OH). The isolated total RNAs were fractionated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a <sup>32</sup>P-labeled probe. All procedures of blot analysis were performed as described previously (Kang et al., 1998).

### Mapping Procedures

An F11 recombinant inbred population, consisting of 164 lines derived from a cross between Milyang 23 and Gihobyeo, was used to construct a molecular map. All mapping procedures were performed as previously reported (Kang et al., 1997; Cho et al., 1998).



### Microscopic Analysis

Rice flowers were fixed in a fixative solution of 50% ethanol, 0.9 M glacial acetic acid, and 3.7% formaldehyde for 15 hr at 4°C, dehydrated with ethanol, infiltrated with xylene, and embedded in paraffin (Paraplast X-tra; Oxford Labware, St. Louis, MO). Twelve-micrometer-thick sections were transferred onto gelatin-coated glass slides, deparaffinized in xylene, and rehydrated in a graded ethanol and water series. The sections were stained in 0.1% toluidine blue O (Sigma), dehydrated with ethanol, infiltrated with xylene, and covered permanently. Light microscopy was performed with a Nikon labophoto-2.

For scanning electron microscopy, the fixed samples were washed with a sodium phosphate buffer, pH 6.8, dehydrated through an ethanol series, and incubated in an ethanol-isoamyl acetate (1:3 [v/v]) mixture for 1 hr. The samples were then dried, mounted on scanning electron microscopy stubs, and coated with gold. The mounted specimens were observed with a scanning electron microscope (model S-4300; Hitachi, Ibaraki-ken, Japan) at an accelerating voltage of 15 kV.

### ACKNOWLEDGMENTS

We thank Hee Jong Koh and Toshiro Kinoshita for providing the *lhs1* seeds and Susan McCouch and Takuji Sasaki for providing rice molecular markers. We thank Ray Wu for providing the rice *act1* promoter and Gi-Hwan Yi for sharing the Dongjin seeds with us. We also thank Chahm An for critical reading of the manuscript; Gurdev Khush, Ilha Lee, and Hee Jong Koh for helpful discussions; and Woong-Suk Yang for technical assistance. This work was supported in part by a grant from the National Research Laboratory Program of Korea Institute of Science and Technology Evaluation and Planning.

Received January 2, 2000; accepted March 27, 2000.

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