Plant Molecular Biology (2005) 59:125–135
DOI 10.1007/s11103-005-2161-y © Springer 2005

Conservation of the E-function for floral organ identity in rice revealed by the analysis of tissue culture-induced loss-of-function mutants of the *OsMADS1* gene

Ganesh Kumar Agrawal, Kiyomi Abe, Muneo Yamazaki, Akio Miyao and Hirohiko Hirochika*
*Plant Functional Genomics Laboratory (PFGL), Molecular Genetics Department, National Institute of Agrobiological Sciences (NIAS), Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan (*author for correspondence; e-mail hirohiko@nias.affrc.go.jp)*

Received 7 April 2004; accepted in revised form 11 February 2005

Key words: flower development, flower meristem, MADS-box, *Oryza sativa* L.

Abstract

Rapid progress in studies on flower development has resulted in refining the classical 'ABC model' into a new 'ABCDE model' to explain properly the regulation of floral organ identity. Conservation of E-function for flower organ identity among the dicotyledonous (dicot) plants has been revealed. However, its conservation in monocotyledonous (monocot) plants remains largely unknown. Here, we show the conservation of E-function in rice (*Oryza sativa* L.) by characterizing tissue culture-induced mutants of two MADS-box genes, *OsMADS1* and *OsMADS5*, which form a subclade within the well-supported clade of *SEP*-genes (E-function) phylogeny. Severe loss-of-function mutations of *OsMADS1* cause complete homeotic conversion of organs (lodicules, stamens, and carpels) of three inner whorls into lemma- and palea-like structures. Such basic deformed structure is reiterated along with the pedicel at the center of the same floret, indicating the loss of determinacy of the flower meristem. These phenotypes resemble the phenotypes caused by mutations of the dicot *E*-class genes, such as the *Arabidopsis SEP123* (*SEPALLATA1/2/3*) and the petunia *FBP2* (*F*loral *B*inding *P*rotein *2*), suggesting that *OsMADS1* play a very similar role in rice to that of defined *E*-class genes in dicot plants. In case of the loss-of-function mutation of *OsMADS5*, no defect in either panicles or vegetative organs was observed. These results demonstrate that *OsMADS1* clearly possesses E-function, and so, E-function is fundamentally conserved between dicot plants and rice, a monocot model plant.

Introduction

Genetic studies of floral homeotic mutants in dicotyledonous (dicot) plants, such as *Arabidopsis thaliana* and *Antirrhinum majus*, have established the 'ABC model' to explain how the identity of the four flower organs (sepals, petals, stamens and carpels) is determined (Bowman *et al*., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Genes involved in determining these functions have been cloned and found mainly to be members of the MADS-box gene family except for *APETALA2* (Bowman *et al*., 1989). Moreover, a wealth of information from distantly related plant species and recent advances in our understanding of the function of MADS-box proteins have modified and expanded the ABC model to the 'ABCDE model' (reviewed in Jack, 2001; Theißen, 2001; Ferrario *et al*., 2004). In this extended ABCDE model, D-function is required for the

ovule development, whereas E-function is needed for the development of petals, stamens and carpels.

E-class genes play fundamental roles in determining the fate of floral development by interacting with, and mediating the expression of, floral organ identity genes (Pelaz *et al*., 2000; Jack, 2001; Theißen, 2001; Ferrario *et al*., 2003, 2004; Malcomber and Kellogg, 2004). *SEP123* (*SEPALLATA1/2/3*) in *Arabidopsis*, *FBP2* (*FLORAL BINDING PROTEIN2*) in petunia, and *TM5* (*T*omato *M*ADS-BOX *5*) in tomato are the typical *E*-class genes (also called *SEP* genes). The *sep123* triple mutant flowers of *Arabidopsis* were found to be indeterminate, where flower organs of inner three whorls were converted into sepals and the 4th whorl was replaced by a new flower composed of only sepals with a pedicel-like structure (Pelaz *et al*., 2000). It was illustrated that the activity of the functionally redundant *SEP123* genes is required for B- and C-functions and to prevent the indeterminate growth of the flower meristem (Pelaz *et al*., 2000). Later, petunia *FBP2* was demonstrated to be the functional equivalent of the *Arabidopsis SEP3* gene in mediating the function of other floral homeotic genes using a combination of transgenic approaches (Ferrario *et al*., 2003). Whereas, classical and refined models are mainly based on studies in dicot plants, studies in monocotyledonous (monocot) plants, such as maize (*Zea mays*; Mena *et al*., 1996; Ambrose *et al*., 2000) and rice (*Oryza sativa* L.; Kang *et al*., 1998; Kyozuka and Shimamoto, 2002; Nagasawa *et al*., 2003), have shown that functions (in particular B- and C-functions) of some of the floral organ identity genes are conserved between monocot and dicot plants. Furthermore, *SEP*-like genes have been identified in monocot plants, but their function is not clear (De Bodt *et al*., 2003; Nam *et al*., 2004). To understand their function in monocot plants, we have characterized *SEP*-like genes of rice.

In rice, there are at least five *SEP*-like genes [*OsMADS1*, *5*, *7* (also called *45*), *8* (also called *24*), *34* (also called *OsMADS19*)] (Malcomber and Kellogg, 2004; Nam *et al*., 2004); for the clarity, *OsMADS45* and *OsMADS24* are mentioned as *OsMADS7* and *OsMADS8*, respectively, throughout the text. These genes belong to at least two subclades, subclade I and II, of well-supported *SEP*-like genes clade (previously called *AGL2*-clade) within the MADS-box gene phylogeny (Figure 1 De Bodt *et al*., 2003; Malcomber and Kellogg, 2004; Nam *et al*., 2004). Subclade II is very unique, as it carries *SEP*-like genes only from monocot plants. To date, *OsMADS1* is the best characterized based on mutations, gene expression, and over-expression (Khush and Librojo, 1985; Chung *et al*., 1994; Jeon *et al*., 2000; Prasad *et al*., 2001; De Bodt *et al*., 2003; Malcomber and Kellogg, 2004). Mutations at amino acid positions 24 and 27 in the MADS domain of *OsMADS1* were found to cause the *l*eafy *h*ull *s*terile *1* (*lhs1*) mutant phenotype in rice (Jeon *et al*., 2000). *lhs1* has leafy lemma and palea, the decreased number of stamens, and, occasionally, an extra pistil or floret (Khush and Librojo, 1985; Jeon *et al*., 2000), implying that *lhs1* mutations affect all four wholes. The mutant phenotype also resembles to the flower phenotype manifested by the transgenic plants over-expressing the *OsMADS1* gene carrying a missense mutation in the MADS domain (Jeon *et al*., 2000). This observed phenotype differs slightly from the reported expression of the *OsMADS1* gene, where it is initially expressed uniformly in young flower primordia and later confined to lemma and palea, with weak expression in carpel (Chung *et al*., 1994; Prasad *et al*., 2001). It is important to note that *lhs1* is a semi-dominant allele, so the mutant phenotype could not be considered as an indication of the *OsMADS1* function (Theißen, 2001; Malcomber and Kellogg, 2004). In another functional study, a role for *OsMADS1* in meristem determinacy was presumed after ectopically expressing the gene in rice and observing that some of the transformed flowers had either reduced or aborted inner floral organs (Prasad *et al*. 2001). Taken together, *OsMADS1* was proposed to act as a B- or C-function gene (Jeon *et al*., 2000; Theißen, 2001; Ferrario *et al*., 2003).

Studies of other rice *SEP*-like genes are mainly based on their gene expression. Expression of *OsMADS5* was detected in stamens and carpel (Kang and An, 1997; De Bodt *et al*., 2003), whereas, the expression of *OsMADS7* and *8* were localized specifically and abundantly in lodicules, developing stamens, and pistil primordia (Greco *et al*., 1997; Pelucchi *et al*., 2002; De Bodt *et al*., 2003). Expression of none of these genes was detected in lemma and palea. *OsMADS34* expression was observed throughout the plant but was turned off in the second and fourth whorls late

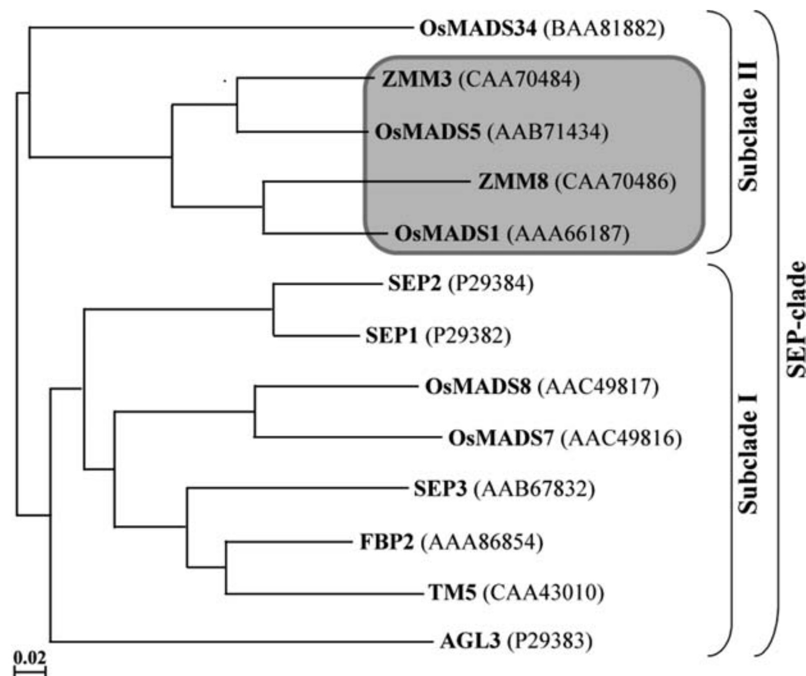

Figure 1. Phylogenetic tree of some members of the *SEP*-like genes from *Arabidopsis*, rice, and maize. Predicted amino acid sequences were used to construct this tree by the neighbor-joining method. *SEP*-like genes form a well-supported clade within the MADS-box gene phylogeny (De Bodt *et al.*, 2003; Nam *et al.*, 2004). Accession numbers are given in the parentheses. *SEP*-clade is further subdivided into subclade I and II. Subclade II (shaded area) is unique, as it is composed of only monocot genes. *SEP1*, *2*, and *3* were previously called *AGL2*, *4*, and *9*, respectively. The scale bar indicates the number of amino acid substitutions per site.

in development (see Malcomber and Kellogg, 2004). *OsMADS7* and *8* are probably functionally redundant, as they have the same expression profile and are classified into the same subclade I (Figure 1). Therefore, the rice *SEP*-like genes have overlapping, but highly variable, expression patterns, suggesting that they might have several functions in flower development of rice. A fine study of *SEP*-like genes in grasses at expression levels supports the above statement (Malcomber and Kellogg, 2004). To get better insight into the function of rice *SEP*-like genes in flower development, we have analyzed loss-of-function mutants of subclade II genes (Figure 1). Evidence presented in this study demonstrates that *OsMADS1* possesses an E-function.

Materials and methods

Plant growth, reverse and forward genetic approaches

Rice (*Oryza sativa* L.) mutant lines were grown in the rice paddy field (Tsukuba, Ibaraki Prefecture, Japan). Recorded day-length during May and August was 14 and 12.4 h, respectively. Reverse genetic analyses were carried out as described elsewhere (reviewed in Hirochika, 2001). In brief, *Tos17* (4114-bp long) specific primers were used in combination with the *OsMADS1* or *OsMADS5* gene specific primers. Two *Tos17* [PA0234 (forward; 5′-ACCACTTCAGAGATTGTGTGGT TGC-3′) and PA0232 (reverse; 5′-CAGCAACGA TGTAGATGGTCAAGC-3′)] and two *OsMADS1* [PA1047 (forward; 5′-CACCATCAG GGTCTTCTCCACC-3′) and PA1048 (reverse; 5′-GTCCATGTAGGCCTGGTGATGG-3′)] or two *OsMADS5* [PA0369 (forward; 5′-TCTAGG GTATGTGCACAGCG-3′) and PA0370 (reverse; 5′-GAGTGAAATTGTGGTTGGGG-3′)] specific primers were used in all possible combinations for polymerase chain reaction (PCR) amplification of genomic DNA from each pooled DNA sample. To exclude non-specific amplified products, we designed two additional *Tos17* [PA0233 (forward; 5′-GACAACACCGGAGCTATACAAATCG-3′) and PA0231 (reverse; 5′-AGGAGGTTGCTTA GCAGTGAAACG-3′)] and *OsMADS1* [PA1049

(forward; 5′-ATCAGGGTGACCATTC CCTGC-3′) and PA1050 (reverse; 5′-GGGCGA ATTATT GTCAGGATTATCG-3′)] or *OsMADS 5* [PA0371 (forward; 5′-AGGGTATGTGCACAG CGCACC-3′) and PA0372 (reverse; (5′-TGAAA TTGTGGTTGGGGGTAAA-3′)] specific primers just upstream of each primer for nested PCR. The amplified DNA fragments were cloned and sequenced to confirm the *Tos17* insertion. For forward genetics, rice mutant lines were screened for defects in floral organs. Samples, such as leaves and spikelets, were collected and stored at appropriate temperature for Southern and Northern analyses. For light microscope and scanning electron microscope (SEM) studies, spikelets were collected freshly before analyses.

Light microscopy and SEM analyses

Spikelets were dissected under light microscopy (Nikon, SMZ-U, Japan). SEM (Model SM-300; TOPCON, Japan) analysis was performed at an accelerating voltage of 15 kV on spikelet without any prior fixation. Wet SEM was carried out according to instructions provided by the manufacturer.

Southern analysis

Rice genomic DNA was isolated from leaves according to the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). Five hundred nanograms of the prepared DNA were digested completely with restriction enzyme *Xba*I, which cuts *Tos17* at one site, and separated on 0.8% (w/v) agarose gel using the Multiblotter MB24 system supplied by Labimap (Plaisir, France). The membrane was prehybridized with Church–Gilbert hybridization buffer (Church and Gilbert, 1984) for 3 h at 65 °C. Probe was added directly to the hybridization buffer, and hybridization was continued for 18 h. Probes were labeled by Multiprime DNA labeling system (Amersham, Buckinghamshire, UK) using [α-^{32}P]dCTP (Amersham), and non-incorporated nucleotides were removed by spin chromatography. The hybridized membrane was washed with $2 \times$ SSC and 0.1% (w/v) SDS (sodium dodecylsulfate) at the same temperature for 1 h, and exposed to an X-ray film (Kodak, Tokyo) for 18 h.

Northern analysis and RT-PCR

Total RNA was isolated from rice flowers using the Isogen kit as per the procedure provided by the manufacturer (Nippon Gene Co., Tokyo). Total RNA (10 μg) was separated on a 1.2% (w/v) formaldehyde-denaturing agarose gels and blotted onto nylon membranes (Hybond N^{+}, Amersham). Prehybridization, hybridization, and probe preparation was done as mentioned above (see 'Southern analysis'). Reverse transcriptase (RT)-PCR was performed using a ProSTAR™ First Strand RT-PCR kit (Stratagene, USA) with the gene specific primers according to the manufacturer's recommendation. A primer pair used for RT-PCR of the *OsMADS1* gene are *OsMADS1*-F: 5′-GCAACTACAACTCACAGGATGC-3′ and *OsMADS1*-R1: 5′-GATGAGCAACCATGTCTG CTGCT-3′.

DNA sequencing and sequence analysis

Nucleotide sequences were determined using an automated DNA sequencer (ABI310 and ABI3100, Applied Biosystems, Foster City, CA) and a BigDye™ Terminator Cycle Sequencing Ready Reaction with either the universal or the gene specific primers. All the sequencing data were analyzed using GENETYX soft-ware (SDC Software Development, Tokyo). The phylogenetic tree was constructed by UPGMA (Unweighted Pair Group Method Arithmetic means).

Results and discussion

Search for Osmads1 mutants

To search for the loss-of-function mutants of *OsMADS1*, we used the tissue culture-induced rice mutant population. Use of this mutant population for identifying the loss-of-function mutants induced by *Tos17* insertion has previously been demonstrated (reviewed in Hirochika, 2001; Kumar and Hirochika, 2001). We applied the PCR-based screening method, using a combination of *OsMADS1*- and *Tos17*-specific primers, and four mutant lines (NC4045, NC7774, ND2920 and NF1019) were identified. Of these, only ND2920 and NF1019 showed altered spikelet structure (Figure 2). Moreover, using a forward genetic

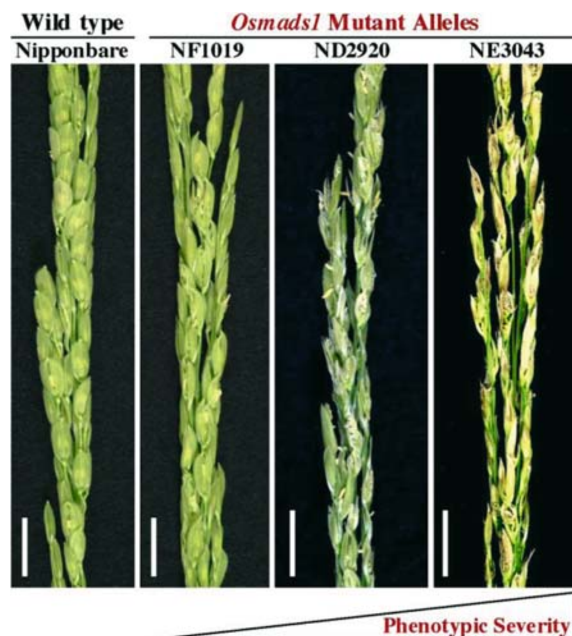

Figure 2. Phenotype of the *Osmads1* mutant alleles. Panicles derived from wild type and *Osmads1* mutant alleles are shown. Allelic mutants are in the order of increasing phenotypic severity. White bars = 1 cm.

approach, two more mutants (NE3043 and NG7558) with phenotypes resembling to those of ND2920 and NF1019 were selected. These mutants were also found to carry mutations in *OsMADS1* (see the following section). These mutant alleles are exhibited in order of the phenotypic severity in Figure 2. As NE3043 and NG7558 had almost identical mutant phenotype, only the NE3043 mutant is presented. The observed phenotypes perfectly co-segregated with the *Osmads1* mutations, as determined by Southern or PCR analyses using more than 100 M_2 plants from each mutant line (data not shown), indicating that the mutant phenotypes are caused by the *Osmads1* mutations. The frequency of *Osmads1* mutant alleles was calculated to be one per three thousand mutant lines.

Phenotypes of the Osmads1 *mutants*

Rice spikelet contains a single floret (consisting of a pistil with a pair of stigma, six stamens, two lodicules, one lemma and one palea) and two glumes (Figures 3A and B). The lodicule, stamen and carpel regions have been assigned as whorls 2, 3 and 4, respectively (reviewed in Ng and Yanofsky, 2001; Theißen, 2001; De Bodt *et al*., 2003). The lemma and palea regions are designated as whorl 1 in this study, though their identity is still uncertain (Ng and Yanofsky, 2001; Theißen, 2001; De Bodt *et al*., 2003). Common phenotypes manifested by the *Osmads1* mutants were under-developed lemma and palea, abnormal flower architecture of a spikelet, delay in flowering time (approximately one week) and sterility. However, the phenotypic severity differed significantly among the mutants (NF1019 being the weakest and NE3043 being the strongest; see Figure 2). The phenotype of NE3043 was almost the same as seen with the NG7558 mutant. No defect in vegetative development was seen. Close inspection of spikelets (Figure 3) derived from the NF1019 mutant revealed the presence of one or more lemmas and paleae (Figure 3C), homeotic transformation of lodicules into leafy lemma- and palea-like structures (Figure 3D; marked by filled arrowheads) and decreased filament length (Figure 3D; marked by open arrowhead). The number of stamens and the presence of carpel varied from spikelet to spikelet, depending on whether spikelet has additional floret. At least 50% of the spikelets possessed additional abnormal floret with an elongated pedicel in the center of the floret (whorl 4), and lodicules as well as stamens were transformed into lemma- and palea-like structures (Figures 3E and F). Most of the spikelets of the ND2920 mutant (an intermediate allele) carried one or more abnormal florets along with pedicel in the center of the floret (Figure 3G). Interestingly, each spikelet of the strongest NE3043 and NG7558 mutants developed multiple abnormal florets, composed of only lemma- and palea-like structures, with pedicel at the center of the same floret (Figures 3H and I). It is noteworthy that none of the spikelets of either the transgenic plants over-expressing the mutant form of *OsMADS1* or the *lhs1* mutants were found to be composed of only lemma- and palea-like structures (Khush and Librojo, 1985; Jeon *et al*., 2000). Moreover, SEM analysis of the terminal part of the mutant spikelet (see Figure 3I; red square) showed the presence of many reiterated lemma- and palea-like structures (Figure 4; Mutant); a few of these are indicated by arrows. These observations indicate that first, these mutants have defects in the function of floral organ identity and determinacy of flower meristem, and second, the flower meristem is indeterminate particularly in the NE3043 and NG7558 mutants.

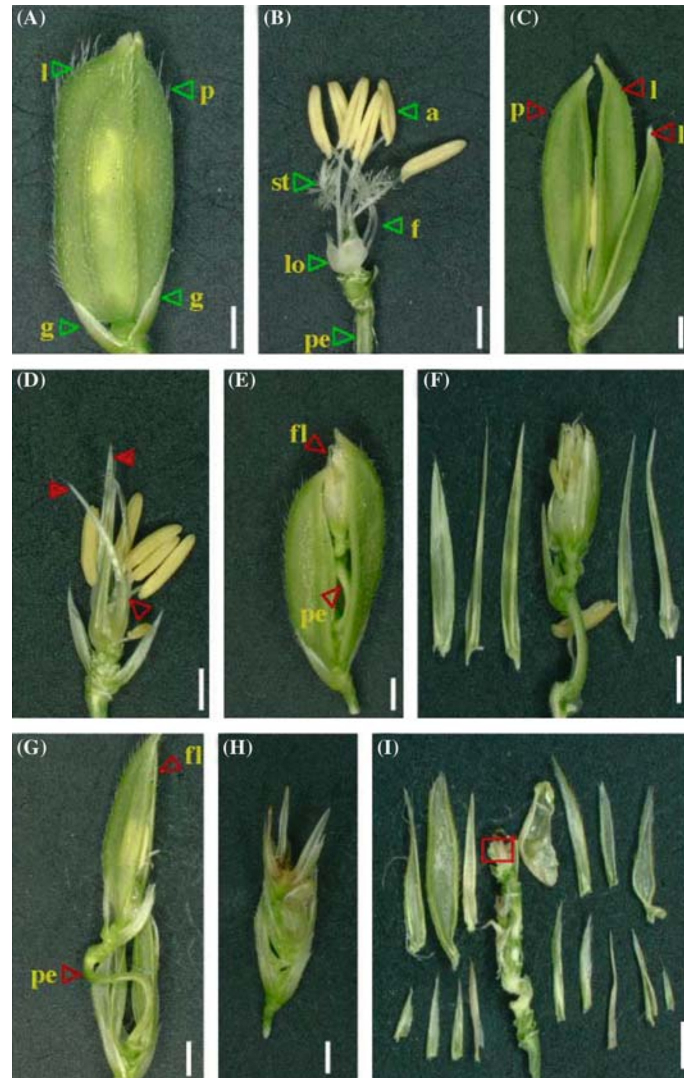

Figure 3. Wild type and mutant spikelets. (A) Wild type spikelet. Lemma (l), palea (p), and glumes (g) are shown. (B) Wild type spikelet after removing lemma, palea and glumes is shown. Normal development of six stamens composed of anthers (a) and filaments (f), two lodicules (lo), a pistil with a pair of stigma (st), and a pedicel (pe) could be seen. (C–F) Individual mutant spikelet shown in C and E are derived from NF1019, and the corresponding dissected mutant flower is shown in D and F, respectively. Filled and open arrowheads in D indicate transformation of lodicules to a leafy lemma/palea and decrease in filament length, respectively. In E, formation of a floret (fl) along with a pedicel (pe) in the spikelet is depicted. Dissection of spikelet, shown in E, reveals an increased number of lemma- and palea-like structures, transformed form of lodicules, and a new flor*et al*ong with pedicel in the center of the spikelet. (G). Spikelet derived from ND2920 represents development of a floret (fl) along with a relatively long pedicel (pe) in the center of the spikelet. (H–I) Spikelet (H), derived from NE3043, unveils reiteration of multiple florets composed of only lemma- and palea-like structures along with pedicel in the spikelet in I. Red square indicates reiterating lemma- and palea-like structures, as shown in Figure 4. White bars = 1 mm.

Molecular causes of the phenotype

Mutations in the mutant lines were analyzed at the molecular level to correlate with the observed phenotype. Analysis of the sequences flanking the *Tos17* insertion indicated its insertion in the 7th and 2th introns of the *OsMADS1* gene in NC4045 and NC7774, respectively (Figure 5A). One possible explanation for no abnormal phenotype in NC4045 and NC7774 mutants is the splicing-out of the *Tos17*-containing intron. Northern analysis, indeed, revealed the presence of the normal

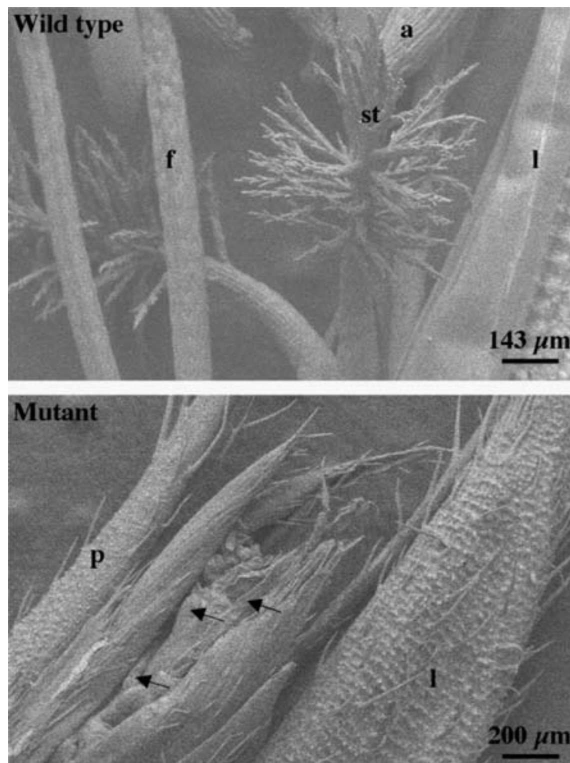

Figure 4. SEM of the wild type and the mutant NE3043. Wild type spikelet carried normal floral organs, such as lemma (l), filament (f), anther (a) and stigma (st). The terminal part of the mutant spikelet (see red square in Figure 3I) revealed a large number of reiterating lemma (l)- and palea (p)-like structures (a few of these marked by arrows), indicating loss of floral determinacy in mutant flowers.

OsMADS1 transcript in both the mutants (data not shown). In NF1019 and ND2920, the *Tos17* insertion was found in the 7th exon of the *OsMADS1* gene, but the direction of its insertion was different (Figure 5A). In the case of NE3043, a deletion of 621-bp (includes 47-bp of the 2nd exon) followed by insertion of 119-bp filler sequence was detected within the *OsMADS1* gene, whereas in NG7558, a small deletion of 39-bp, including 20-bp of the 6th exon and 19-bp of the 6th intron, was found (indicated by red in Figure 5A).

We further examined the *OsMADS1* expression in spikelets of the wild type and mutants by Northern analysis using *OsMADS1* gene probe specific to 3′-UTR (untranslated region) (Figure 5B). As expected, a single 1.2-kb transcript was detected in the wild type (W) but not in the mutant (M). However, a very faint transcript of approximately 1.1-kb in NF1019 and two clear but overlapping transcripts of 1.25- and 1.3-kb in NG7558 were observed. Such transcripts were reasoned to be products of alternative splicing. To confirm this, RT-PCR was performed on the cDNA synthesized from the same total RNA used for northern analysis. A product of an expected size of 0.7-kb was obtained in the wild type but a fragment of altered size in NF1019 (0.6-kb) and NG7558 (0.8-kb) mutants (data not shown). The calculated differences of the RTPCR products between the wild type and mutants of NF1019 and NG7558 matched well with the transcript size shown by northern analysis (Figure 5B). Sequence analysis of the PCR products revealed the deletion of 114-bp (corresponding to 38-aa; starting from ^{183}Gln to ^{220}Gly in the C-terminal domain) in NF1019. The 0.8-kb RT-PCR product was found to carry the sequences from the 6th intron (data not shown). Therefore, the large transcripts in the NG7558 mutant might be due to alternative splicing. In all, these results illustrate that abnormal spikelets manifested by the *Osmads1* mutant alleles are due to loss of the normal *OsMADS1* transcript.

Phenotypic severity is most likely associated with the mutation position

As mentioned above, phenotypic severity differs among the *Osmads1* mutant alleles (see Figures 2 and 3). In light of our findings, we suspect that phenotypic severity may be associated with the location of mutations on the gene and the presence of alternatively spliced *OsMADS1* transcript(s). It appears that the mutation within N-terminal region of OsMADS1 confers stronger phenotype (NE3043 and NG7558) than the mutation within the C-terminal region (NF1019 and ND2920). There are precedents from other plants that the position of the mutation in a gene has a significant effect on phenotype (Chuck *et al.*, 2002). In case of NF1019, an alternatively spliced transcript of 1.1-kb, which can encode a protein with 38-aa deletion (^{183}Gln to ^{220}Glyin the C-terminal domain), may also account for being the weakest *Osmads1* allele. It is important to mention that NE3043 and NG7558 could be considered as null mutants based on their phenotypic severity, mutation position, and loss of the *OsMADS1* normal transcript. Furthermore, observation of the similar

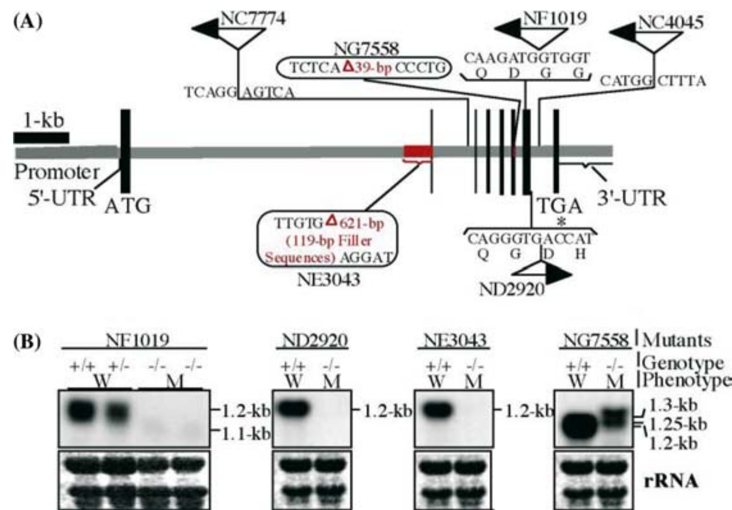

Figure 5. (A) Genomic organization of *OsMADS1* and mutations in its mutant alleles. The *OsMADS1* gene contains seven introns and eight exons (black vertical boxes) along with its promoter, 5′-UTR, and 3′-UTR. Molecular changes other than *Tos17*-insertions are presented in red characters. Arrows represent direction of the *Tos17* insertion, but do not reflect the actual size of *Tos17* (4114-bp). A few nucleotides surrounding the mutation positions have been presented to designate the mutation position. (B) Northern analysis of *OsMADS1* in the spikelets of wild type (W) and mutant (M) plants. Total RNA (10 μg) was subjected to Northern analysis and hybridized with the 3′-UTR specific probe. Equal loading was confirmed by staining the membrane with methylene blue after blotting, and ribosomal RNA bands (shown as rRNA) are presented. Transcript size is shown at the right hand side. *Osmads1* allelic mutants, genotype and phenotype are also mentioned.

syndrome in at least these two independent *Osmads1* mutant lines (NE3043 and NG7558) indicates that phenotypic alterations are gene-specific.

Loss of B- and C-function in the Osmads1 *mutants*

The syndrome conferred by the *Osmads1* mutations is distinguishable from that of the homeotic alterations caused by B- or C-function genes in rice and maize. In rice, homologs of B (*OsMADS2* and *OsMADS4* are homologous to *PISTILLATA* whereas *OsMADS16* is homologous to *APETALA3*)- and *C* (*OsMADS3* is homologous to *AGAMOUS*)-function genes have been cloned and characterized (Kang *et al*., 1998; Kyozuka and Shimamoto, 2002; Nagasawa *et al*., 2003). Co-suppression of *OsMADS3* in rice resulted in development of lodicule-like organs in whorl 3 and several abnormal flowers in whorl 4, whereas transgenic rice plants expressing antisense of *OsMADS4* showed transformation of lodicules resembling lemma/palea, and stamens to carpels (Kang *et al*., 1998). On the other hand, ectopic expression of *OsMADS3* caused a homeotic transformation of lodicules to stamens (Kyozuka and Shimamoto, 2002). *OsMADS16* mutants, called *SUPERWOMAN1*, manifested transformation of stamens and lodicules into carpels- and palea-like organs, respectively (Nagasawa *et al*., 2003). In maize, mutants of the *B* (*SILKY1*) and *C* (*ZAG1*) genes have been extensively studied. The *silky1* mutant showed a homeotic conversion of lodicules and stamens into palealike organ and carpels, respectively (Ambrose *et al*., 2000). The *zag1* mutants revealed loss of determinacy but no defects in organ identity (Mena *et al*., 1996). Moreover, the *silky1 zag1* double mutants developed repeated whorls of lemma/palea-like organs. In this context, the severe phenotype conferred by the *Osmads1* mutants (NE3043 and NG7558) can be interpreted as if the mutants have lost both the B- and C-homeotic functions. These results suggest that *OsMADS1* is required for the proper functioning of both B- and C-functions pathways.

E-function in rice

The mode of expression and the pattern of defects incurred by loss-of-function of the *OsMADS1* gene clearly indicate that *OsMADS1* is a novel player involved in determination of both the floral

organ identity and the meristem determinacy. The characteristic phenotypes of the *Osmads1* mutants (NE3043 and NG7558) – conversion of inner three whorls into lemma- and palea-like structures and replacement of the 4th whorl by a new flower composed of only lemmas and paleae with a pedicel-like structure – resemble the *Arabidopsis sep123* triple mutant, a well-defined E-function mutant (reviewed in Jack, 2001; Theißen, 2001; Ferrario *et al.*, 2004). Furthermore, like *SEP3*, *OsMADS1* is also involved in determining the flowering time. Constitutive expression of *SEP3* in *Arabidopsis* plants caused early flowering (Pelaz *et al.*, 2001). Similarly, ectopic expression of *OsMADS1* in both tobacco and rice also triggered an early flowering, approximately by a week, compared to that of the wild type plants (Chung *et al.*, 1994; Jeon *et al.*, 2000). In line with these findings, we recorded that *Osmads1* mutants delay the flowering time, approximately by a week. In summary, *OsMADS1* has functions very similar to that of the defined *SEP*-function in *Arabidopsis*. Therefore, *OsMADS1* has an E-function in rice.

Interestingly, *OsMADS1* does not have a simple genetic orthologous relationship with the *Arabidopsis SEP* genes (Figure 1). One possible reason is that the genes of subclade II may have acquired additional function(s) during the course of evolution. The mutant phenotype caused by the loss-of-function of *OsMADS1* alone supports our idea, because to confer the similar phenotype in *Arabidopsis*, three functionally redundant *SEP* genes must be knocked out (Pelaz *et al.*, 2000). Recently, in an elegant study on examination of the expression of *SEP*-like genes in several distantly related grass species, including rice and maize, it was assumed that *SEP*-like genes may have affected morphological diversification of grass inflorescences by mediating the expression of different floral identity genes in different regions of the floret and spikelet (Malcomber and Kellogg, 2004). This seems to be the case, as we discuss below that *OsMADS1* may be also involved in controlling the identities of lemma and palea in rice.

OsMADS1 *may be involved in development of lemma and palea*

The lemma and palea have unique function in rice, and that is to protect the seed from any damaging environmental factors by covering it properly. Under-developed lemma and palea are one of the common features observed in all the *Osmads1* mutant alleles. Though we do not consider this feature as a homeotic function, we believe that in addition to E-function, *OsMADS1* may also have a role in the development of lemma and palea. This is likely, given the fact that first, *OsMADS1* is strongly expressed in the lemma and palea at the later stage of the flower development (Chung *et al.*, 1994; De Bodt *et al.*, 2003), and second, OsMADS1 physically interacts with AP1-like proteins, such as OsMADS14, 15 and RAP1B (Moon *et al.*, 1999; Lim *et al.* 2000; Cooper *et al.*, 2003). AP1, an *Arabidopsis* A-function protein, determines the sepal identity (Bowman *et al.*, 1989). Moreover, previous studies on *OsMADS1* mutations and over-expression have reported that leafy lemma and palea, and thus the open flower, are the remarkable features observed in all flowers (Jeon *et al.*, 2000; Prasad *et al.*, 2001). In *Arabidopsis*, the *sep123* triple mutant manifested almost normal sepal development (Pelaz *et al.*, 2000). This difference might be due to, first, the low level expression of *SEP3* in the sepals (Mandel and Yanofsky, 1998), and second, the differences in the genetic control of lemma and palea development compared to the dicot sepals.

Functional analysis of OsMADS5 *– the nearest relative of* OsMADS1

To know the functional significance of *OsMADS5*, sharing 72% identity with *OsMADS1* at amino acid level (Kang *et al.*, 1997), in flower development, we searched for its loss-of-function mutants. Using PCR-based reverse genetics approach, two mutant lines (NC2009 and NC5003) of *OsMADS5* were identified. Analysis of sequences flanking the *Tos17* insertion revealed its insertion in the 2nd intron and 4th exon of the *OsMADS5* gene in NC2009 and NC5003, respectively (Figure 6A). To know the *OsMADS5* expression in the mutants, RT-PCR was carried out on cDNA derived from total RNA of the W and M spikelets. We used RT-PCR considering the very low level expression of *OsMADS5* in flower organs. As expected, RT-PCR product of expected size (959-bp) was detected in both the wild type and

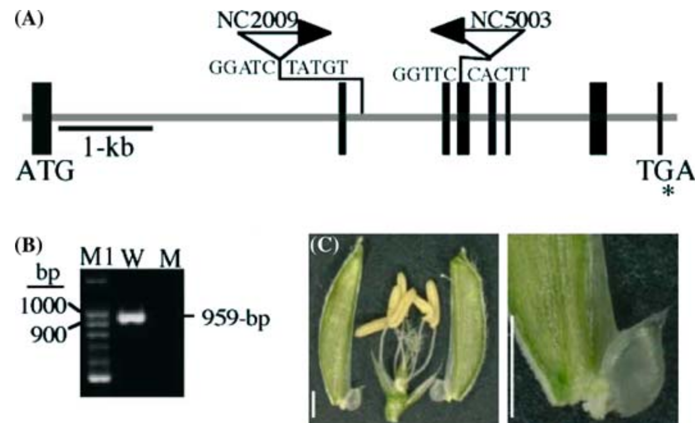

Figure 6. (A) Genomic organization of *OsMADS5* and location of *Tos17*-insertions in its mutant alleles. *OsMADS5* is composed of seven introns and eight exons (black boxes). *Tos17*-insertions (shown by arrows representing direction) and a few nucleotides surrounding the insertion sites are presented. (B) RT-PCR was performed on cDNA derived from spikelets of wild type (W) and mutant NC5003 (M) using the *OsMADS5* 5′ (OsMADS5-F: 5′-GGAGAGGGAGAGAGAGGGAA-3′)- and 3′ (OsMADS5-R: 5′-ATTCATCAATCAAACCGGGA-3′)-UTR specific primers. M1 represents the 100-bp DNA ladder. (C) The phenotype of the NC5003 mutant is an attachment of lodicule to lemma and palea. White bars = 1 mm.

mutant of NC2009 (data not shown). However in NC5003, no RT-PCR product was seen in the mutant, whereas 959-bp RT-PCR product was detected in the wild type (Figure 6B). These results indicate that the *Tos17* insertion in NC5003 has resulted in the loss-of-function of the *OsMADS5* gene. A visual observation of the *Osmads5* plants failed to detect any abnormality in spikelets and in plants as a whole. However, careful observations of the florets showed slightly deformed structure of lodicules (Figure 6C). Lodicules were found to attach with the lemma and palea. These results suggest that *Osmads5* mutation has almost no effect on flower development. Therefore, in the subclade II, *OsMADS1* is the gene that possesses an E-function.

In conclusion, we have carried out functional analysis of rice *SEP*-like genes of subclade II, and have demonstrated that *OsMADS1* has evidently the E-function in rice, revealing the conservation of E-function between rice and dicot plants. Moreover, like some *SEP* genes with divergent functions (Kotilainen *et al*., 2000; Pelaz *et al*., 2000; Tzeng *et al*., 2003), *OsMADS1* is likely to participate in specifying the identities of lemma and palea. These findings raise important questions. What is the functional role of *OsMADS7* and *OsMADS8*, which are most closely related with the *Arabidopsis SEP* genes? Do they function by interacting with *OsMADS1*?

Acknowledgements

We thank Drs Masaki Mori and Hiroshi Takatsuji for providing the SEM facility and critical reading the manuscript, respectively. This work was supported by a project grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Rice Genome Project MP-2102), a grant for the enhancement of center-of-excellence, the special co-ordination funds for promoting Science and Technology in Japan, and the Program for Promotion of Basic Research Activities for Innovative Biosciences (BRAIN). GKA was supported by JSPS (Japan Society for the Promotion of Science).

References

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F. and Schmidt, R.J. 2000. Molecular and genetic analyses of the *silky* gene reveal conservation in floral organ specification between eudicots and monocots. Mol. Cell 5: 569–579.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. 1989. Genes directing flower development in *Arabidopsis*. Plant Cell 1: 37–52.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. 1991. Genetic interactions among floral homeotic genes of *Arabidopsis*. Development 112: 1–20.

Chuck, G., Muszynski, M., Kellogg, E., Hake, S. and Schmidt, R.J. 2002. The control of spikelet meristem identity by the *branched silked1* gene in maize. Science 298: 1238–1241.

Chung, Y.-Y., Kim, S.-R., Finkel, D., Yanofsky, M.F. and An, G. 1994. Early flowering and reduced apical dominance result from ectopic expression of a rice MADS box gene. Plant Mol. Biol. 26: 657–665.

Church, G.M. and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81: 1991–1995.

Coen, E.S. and Meyerowitz, E.M. 1991. The war of the whorls: genetic interactions controlling flower development. Nature 353: 31–37.

Cooper, B., Clarke, J.D., Budworth, P., Kreps, J., Hutchison, D., Park, S., Guimil, S., Dunn, M., Luginbuhl, P., Ellero, C., Goff, S.A. and Glazebrook, J. 2003. A network of rice genes associated with stress response and seed development. Proc. Natl. Acad. Sci. USA 100: 4945–4950.

De Bodt, S., Raes, J., Van de Peer, Y. and Theißen, G. 2003. And then there were many: MADS goes genomic. Trends Plant Sci. 8: 475–483.

Ferrario, S., Immink, R.G.H., Shchennikova, A., Busscher-Lange, J. and Angenent, G.C. 2003. The MADS box gene *FBP2* is required for SEPALLATA function in petunia. Plant Cell 15: 914–925.

Ferrario, S., Immink, R.G.H. and Angenent, G.C. 2004. Conservation and diversity in flower land. Curr. Opin. Plant Biol. 7: 84–91.

Greco, R., Stagi, L., Colombo, L., Angenent, G.C., Sari-Gorla, M. and Pe, M.E. 1997. MADS box genes expressed in developing inflorescences of rice and sorghum. Mol. Gen. Genet. 253: 615–623.

Hirochika, H. 2001. Contribution of the *Tos17* retrotransposon to rice functional genomics. Curr. Opin. Plant Biol. 4: 118–122.

Jack, T. 2001. Relearning our ABCs: new twists on an old model. Trends Plant Sci. 6: 310–316.

Jeon, J.-S., Jang, S., Lee, S., Nam, J., Kim, C., Lee, S.-H., Chung, Y.-Y., Kim, S.-R., Lee, Y.H., Cho, Y.-G. and An, G. 2000. *leafy hull sterile1* is a homeotic mutation in a rice MADS box gene affecting rice flower development. Plant Cell 12: 871–884.

Kang, H.-G. and An, G. 1997. Isolation and characterization of a rice MADS box gene belonging to the *AGL2* gene family. Mol. Cells 7: 45–51.

Kang, H.-G., Jang, S., Chung, J.-E., Cho, Y.-G. and An, G. 1997. Characterization of two rice MADS box genes that control flowering time. Mol. Cells 7: 559–566.

Kang, H.-G., Jeon, J.S., Lee, S. and An, G. 1998. Identification of class B and class C floral organ identity genes from rice plants. Plant Mol. Biol. 38: 1021–1029.

Khush, G.S. and Librojo, A.L. 1985. Naked seed rice (NSR) is allelic to *op* and *lhs*. Rice Genet. News 2: 71.

Kotilainen, M., Elomaa, P., Uimari, A., Albert, V.A., Yu, D.Y. and Teeri, T.H. 2000. *GRCD1*, an *AGL2*-like MADS box gene, participates in the C function during stamen development in Gerbera hybrida. Plant Cell 12: 1893–1902.

Kumar, A. and Hirochika, H. 2001. Applications of retrotransposons as genetic tools in plant biology. Trends Plant Sci. 6: 127–134.

Kyozuka, J. and Shimamoto, K. 2002. Ectopic expression of *OsMADS3*, a rice ortholog of *AGAMOUS*, caused a homeotic transformation of lodicules to stamens in transgenic rice plants. Plant Cell Physiol. 43: 130–135.

Lim, J., Moon, Y.-H., An, G. and Jang, S.K. 2000. Two rice MADS domain proteins interact with *OsMADS1*. Plant Mol. Biol. 44: 513–527.

Malcomber, S.T. and Kellogg, E.A. 2004. Heterogeneous expression patterns and separate roles of the *SEPALLATA* gene *LEAFY HULL STERILE1* in grasses. Plant Cell 16: 1692–1706.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F. and Schmidt, R.J. 1996. Diversification of C-function activity in maize flower development. Science 274: 1537–1540.

Moon, Y.-H., Kang, H.-G., Jung, J.-Y., Jeon, J.-S., Sung, S.-K. and An, G. 1999. Determination of the motif responsible for interaction between the rice APETALA1/AGAMOUS-LIKE9 family proteins using a yeast two-hybrid system. Plant Physiol. 120: 1193–1204.

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant

DNA. Nucl. Acids Res. 8: 4321.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H.-Y., Sakai, H. and Nagato, Y. 2003. *SUPERWOMAN1 and DROOPING LEAF* genes control floral organ identity in rice. Development 130: 705–718.

Nam, J., Kim, J., Lee, S., An, G., Ma, H. and Nei, M. 2004. Type I MADS-box genes have experienced faster birth-and death evolution than II MADS-box genes in angiosperms. Proc. Natl. Acad. Sci. USA 101: 1910–1915.

Ng, M. and Yanofsky, M.F. 2001. Function and evolution of the plant MADS-box gene family. Nature Rev. Genet. 2: 186–195.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E., Yanofsky, M.F. 2000. B and C floral organ identity functions require *SEPALLATA* MADS-box gene. Nature 405: 200–203.

Pelaz, S., Gustafson-Brown, C., Kohalmi, S.E., Crosby, W.L. and Yanofsky, M.F. 2001. *APETALA1* and *SEPALLATA3* interact to promote flower development. Plant J. 26: 385–394.

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., Pe, M.E., Colombo, L. and Kater, M.M. 2002. Comparative analysis of rice MADS-box genes expressed during flower development. Sex. Plant Rep. 15: 113–122.

Prasad, K., Sriram, P., Kumar, C.S., Kushalappa, K. and Vijayraghavan, U. 2001. Ectopic expression of rice *OsMADS1* reveals a role in specifying the lemma and palea, grass floral organs analogous to sepals. Dev. Genes Evol. 211: 281–290.

Theißen, G. 2001. Development of floral organ identity: stories from the MADS house. Curr. Opin. Plant Sci. 4: 75–85.

Tzeng, T.Y., Hsiao, C.C., Chi, P.J. and Yang, C.H. 2003. Two lily SEPALLATA-like genes cause different effects on floral formation and floral transition in *Arabidopsis*. Plant Physiol. 133: 1091–1101.

Weigel, D. and Meyerowitz, E.M. 1994. The ABCs of floral homeotic genes. Cell 78: 203–209.