the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2010) **61**, 767–781 doi: 10.1111/j.1365-313X.2009.04101.x

Functional conservation and diversification of class E floral homeotic genes in rice (*Oryza sativa*)

Rongfeng Cui[1,2,†], Jiakun Han[1,2,†], Suzhen Zhao[1,†], Kunmei Su[1,†], Feng Wu[1], Xiaoqiu Du[1], Qijiang Xu[1], Kang Chong[1], Günter Theißen[3,*] and Zheng Meng[1,*]

[1]*Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China,*

[2]*Graduate School, Chinese Academy of Sciences, Beijing 100039, China, and*

[3]*Department of Genetics, Friedrich Schiller University Jena, D-07743 Jena, Germany*

Received 16 October 2009; revised 23 November 2009; accepted 27 November 2009; published online 22 January 2010.
[*]For correspondence (fax +49 3641 949 552; e-mail guenter.theissen@uni-jena.de; fax +86 1082 599 701; e-mail zhmeng@ibcas.ac.cn).
[†]These authors contributed equally to this work.

SUMMARY

Mutant analyses in different eudicotyledonous flowering plants demonstrated that *SEPALLATA*-like MADS-box genes are required for the specification of sepals, petals, stamens and carpels, and for floral determinacy, thus defining class E floral organ identity genes. *SEP*-like genes encode MADS-domain transcription factors and constitute an angiosperm-specific gene clade whose members show remarkably different degrees of redundancy and sub-functionalization within eudicots. To better understand the evolutionary dynamics of *SEP*-like genes throughout the angiosperms we have knocked down *SEP*-like genes of rice (*Oryza sativa*), a distant relative of eudicots within the flowering plants. Plants affected in both *OsMADS7* and *OsMADS8* show severe phenotypes including late flowering, homeotic changes of lodicules, stamens and carpels into palea/lemma-like organs, and a loss of floral determinacy. Simultaneous knockdown of the four rice *SEP*-like genes *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8*, leads to homeotic transformation of all floral organs except the lemma into leaf-like organs. This mimics the phenotype observed with the *sep1 sep2 sep3 sep4* quadruple mutant of *Arabidopsis*. Detailed analyses of the spatial and temporal mRNA expression and protein interaction patterns corresponding to the different rice *SEP*-like genes show strong similarities, but also gene-specific differences. These findings reveal conservation of *SEP*-like genes in specifying floral determinacy and organ identities since the separation of eudicots and monocots about 150 million years ago. However, they indicate also monocot-specific neo- and sub-functionalization events and hence underscore the evolutionary dynamics of *SEP*-like genes. Moreover, our findings corroborate the view that the lodicules of grasses are homologous to eudicot petals.

Keywords: *SEPALLATA*, class E floral organ identity genes, rice, *Oryza sativa*, flower development.

INTRODUCTION

Flowering plants (angiosperms) have evolved a tremendous diversity of floral structures since they originated about 200 million years ago (Wikstrom *et al.*, 2001; Soltis and Soltis, 2004; Endress, 2006). The elucidation of developmental genetic pathways in eudicot model species such as *Arabidopsis thaliana*, *Petunia hybrida* and *Antirrhinum majus* has provided ample evidence that changes in the function of key regulatory genes contributed significantly to the evolution of new morphologies (Cronk, 2001; Frohlich, 2003; Irish, 2003).

Previous studies have shown that orthologous genes from different taxa can display divergent functions, which may provide the genetic basis for the floral diversification among flowering plants (Theissen *et al.*, 2000; Irish and Litt, 2005; Soltis *et al.*, 2007; Theissen and Melzer, 2007). Thus comprehensive comparative developmental studies of floral homeotic genes in diverse taxa are needed to better understand the evolutionary origin and subsequent diversification of flowers (Baum *et al.*, 2002).

Based on genetic analyses of homeotic mutants primarily in Arabidopsis and Antirrhinum (Carpenter and Coen, 1990; Schwarz-Sommer *et al.*, 1990; Bowman *et al.*, 1991), the ABC model was proposed to explain the determination of floral

organ identities (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). According to this model, class A genes specify the identity of sepals, class A and B genes specify petal identity, class B and C genes determine stamen identity, and class C genes determine carpel identity. Recently, reverse genetic studies demonstrated that two additional classes of floral homeotic genes, termed class D and class E genes, are also necessary for the specification of floral organ identity. While class D genes are crucial for ovule development (Colombo *et al.*, 1995; Favaro *et al.*, 2003; Pinyopich *et al.*, 2003), class E genes are required for the specification of all kinds of floral organs (Pelaz *et al.*, 2000; Honma and Goto, 2001; Ditta *et al.*, 2004). Based on these findings, the 'ABCDE model' was proposed to explain how the different floral organ identity genes interact during the determination of floral organs (Theissen, 2001).

In the model plant *Arabidopsis*, class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*) (Mandel *et al.*, 1992; Jofuku *et al.*, 1994), class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Goto and Meyerowitz, 1994; Jack *et al.*, 1992), the class C gene by *AGAMOUS* (Yanofsky *et al.*, 1990), class D genes by *SEEDSTICK* (*STK*; formerly *AGL11*), *SHATTERPROOF1* (*SHP1*; *AGL1*) and *SHP2* (*AGL5*) (Savidge *et al.*, 1995), and class E genes by *SEPALLATA1*, *2*, *3* and *4* (*SEP1*, *2*, *3* and *4*; previously known as *AGL2*, *4*, *9* and *3*, respectively) (Ma *et al.*, 1991; Huang *et al.*, 1995; Mandel and Yanofsky, 1998; Pelaz *et al.*, 2000; Ditta *et al.*, 2004). Except for *AP2*, all class A, B, C, D, and E floral homeotic genes are $MIKC^c$-type MADS-box genes, named after their conserved structure comprising an M (MADS), I (Intervening), K (Keratin-like) and C (C-terminal) domain (Riechmann and Meyerowitz, 1997; Egea-Cortines *et al.*, 1999; Becker *et al.*, 2000; Theissen *et al.*, 2000; Honma and Goto, 2001). These genes encode proteins acting as transcription factors that determine floral organ identities by binding to *cis*-regulatory elements of target genes termed 'CArG-boxes' (consensus 5′-$CC(A/T)_6GG$-3′) (Tröbner *et al.*, 1992; Riechmann *et al.*, 1996).

In the *Arabidopsis sep1/2/3* triple loss-of-function mutant, all petals, stamens and carpels are converted into sepal-like organs, and flower development becomes indeterminate; thus the phenotype of *sep1/2/3* triple mutants largely resembles the phenotype of class *bc* (i.e. *ap3 ag* or *pi ag*) double mutants (Pelaz *et al.*, 2000, 2001; Honma and Goto, 2001). In the *sep1/2/3/4* quadruple mutant, all flower organs are converted into organs resembling vegetative leaves, are arranged in spiral phyllotaxis and again, appear in indeterminate number, thus resembling class *abc* triple loss-of-function mutants (Ditta *et al.*, 2004). In the *sep* mutants, however, the early expression of the class A, B and C floral homeotic genes is not affected. These findings indicate that some *SEP*-like (*AGL2*-like) genes are required for class A, B and C gene functions in the specification of flower organ identity and in conferring determinate growth to the flower, but they are not just activators of class ABC genes, but a new class of organ identity genes. Therefore, they were added as 'class E genes' to the ABC model (Theissen, 2001).

Phylogenetic analyses revealed that *SEP*-like genes are monophyletic, and that they can be divided into two subclades, *SEP1/2/4*-like genes (*AGL2/3/4* clade *sensu*) (Zahn *et al.*, 2005) and *SEP3*-like genes (*AGL9* clade), which were generated by a gene duplication (or whole genome duplication) that probably predated the origin of the most recent common ancestor of extant angiosperms (Theissen *et al.*, 1996; Becker and Theissen, 2003; Zahn *et al.*, 2005). As *SEP*-like genes are required for specifying the 'floral state' by contributing to floral organ and meristem identity (Zahn *et al.*, 2005), they may have played a critical role during the evolutionary origin of the flower and are thus of utmost evolutionary interest.

Despite their evolutionary importance, due to technical limitations the function of few *SEP*-like genes outside of *Arabidopsis* have already been studied, including genes from core eudicots, such as tomato (*Solanum lycopersicum*) (Pnueli *et al.*, 1994; Ampomah-Dwamena *et al.*, 2002), petunia (*Petunia hybrida*) (Angenent *et al.*, 1994), and gerbera (*Gerbera hybrida*) (Kotilainen *et al.*, 2000; Uimari *et al.*, 2004). These investigations suggested a general conservation of *SEP*-like gene function in eudicots. However, two *Gerbera SEP*-like genes, *GERBERA REGULATOR OF CAPITULUM DEVELOPMENT1* (*GRCD1*) and *GRCD2*, revealed whorl-specific subfunctionalization, with *GRCD2* acting in whorl 4 and *GRCD1* acting in whorl 3 (Kotilainen *et al.*, 2000; Uimari *et al.*, 2004). Furthermore, *GRCD2* has been found to play a role in regulating inflorescence development in addition to flower development (Uimari *et al.*, 2004).

Quite a number of *SEP*-like genes have also been identified from monocots. In maize there are at least eight different *SEP*-like genes with distinguishable expression patterns suggesting diverse functions, which most likely reflects the evolution of complex inflorescence structures, at least in part (Theissen *et al.*, 1996; Cacharron *et al.*, 1999; Becker and Theissen, 2003; Zahn *et al.*, 2005). In rice there are five different *SEP*-like genes (Arora *et al.*, 2007), with *OsMADS1* (also known as *LEAFY HULL STERILE1*, *LHS1* and *NAKED SEED RICE*, *NSR* (Chen *et al.*, 2006; Jeon *et al.*, 2000), *OsMADS5* and *OsMADS34* (also called *OsMADS19*) probably being members of the *SEP1/2/4* clade of genes, and *OsMADS7* and *OsMADS8* (also known as *OsMADS45* and *OsMADS24*, respectively) being *SEP3*-like genes (Kang *et al.*, 1997; Zahn *et al.*, 2005).

The functions of monocot *SEP*-like genes are unknown, except for *OsMADS1*, for which a class E gene function has been described (Agrawal *et al.*, 2005; Prasad *et al.*, 2005). As there is evidence that *SEP3* is more important for the class E gene function than any of the other three *SEP* genes in *Arabidopsis* (reviewed by Melzer *et al.*, 2009) this is remarkable, given that *OsMADS7* and *OsMADS8* appear to be more

similar to *SEP3* than *OsMADS1* in terms of phylogenetic relationship and expression patterns (Münster *et al.*, 2002; Becker and Theissen, 2003; Malcomber and Kellogg, 2004, 2005; Nam *et al.*, 2004; Prasad *et al.*, 2005). Thus a *priori* *OsMADS7* and *OsMADS8* may have appeared more likely candidates for providing the class E gene function than *OsMADS1*, and so the question arises as to what functions the other four rice *SEP*-like genes other than *OsMADS1* have. As rice is only quite distantly related to *Arabidopsis* within the angiosperms, answering that question may tell us a great deal about the evolutionary conservation and dynamics of the *SEP*-like genes and class E floral homeotic gene function.

Therefore, we explored the functions of the *SEP*-like genes of rice by a reverse genetics approach employing double-stranded RNA-mediated interference (Chuang and Meyerowitz, 2000; Baulcombe, 2002; Hannon, 2002) by silencing different *SEP*-like genes individually and in combination, and studying the expression of these genes and the interactions of the proteins encoded by them. Our data demonstrate conservation of *SEP*-like genes in specifying floral determinacy and organ identities since the separation of eudicots and monocots about 150 million years age, but also reveal monocot-specific neo- and sub-functionalization events, thus indicating an unexpected evolutionary dynamics of *SEP*-like genes.

RESULTS

Silencing of either *OsMADS7* or *OsMADS8* produces only mild mutant phenotypes

Sixteen independent transgenic lines transformed with pUJOsM7C, named *OsM7*-RNAi, and 46 independent transgenic lines with the pUJOsM8 construct, termed *OsM8*-RNAi, were generated. Real-time PCR revealed that expression of the corresponding genes (*OsMADS7* in the *OsM7*-RNAi lines and *OsMADS8* in the *OsM8*-RNAi lines) was specifically down-regulated, while the expression of other *SEP*-like genes was surprisingly upregulated compared with the wild type (Fig. 1a). In a relatively high number of flowers of the *OsMADS7* knockdown lines a carpel with three stigmas was found (Fig. S1, Table 1). No other obvious alterations were observed in either vegetative or reproductive organs of all these lines. As both the sequences and expression patterns of *OsMADS7* and *OsMADS8* are quite similar we assumed that functional redundancy between *OsMADS7* and *OsMADS8* might be one reason why the observed mutant phenotypes deviate only weakly, at best, from the wild type.

Silencing of both *OsMADS7* and *OsMADS8* causes severe morphological alterations of floral organs

To test for functional redundancy between *OsMADS7* and *OsMADS8* we silenced both genes simultaneously,

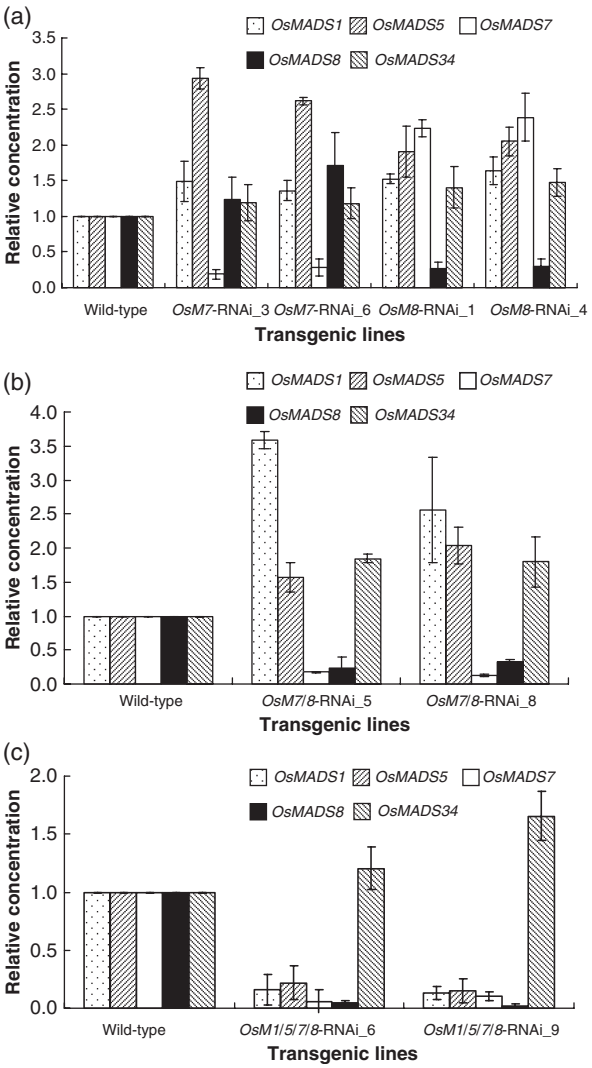

Figure 1. Molecular characterization of RNAi transgenic plants for rice *SEP*-like genes.
(a) Real-time PCR analyses to evaluate the expressions of *OsMADS7*, *OsMADS8*, *OsMADS1*, *OsMADS5* and *OsMADS34* in the *OsM7*-RNAi and *OsM8*-RNAi transgenic lines.
(b) Real-time PCR analyses to evaluate the expressions of *OsMADS7*, *OsMADS8*, *OsMADS1*, *OsMADS5* and *OsMADS34* in the *OsM7/8*-RNAi transgenic lines.
(c) Real-time PCR analyses of two independent lines transformed with the pUJOsM8M construct.

employing pUJOsM7I. This vector contains the region from nucleotides 149 to 454 (corresponding to the translation start codon ATG at 1) of the *OsMADS7* coding sequence, which contains 29 nucleotides that are identical between *OsMADS7* and *OsMADS8* (Table S1). 18 independent pUJOsM7I transgenic lines were generated. The expression levels of all five *SEP*-like genes in the transgenic lines (named *OsM7/8*-RNAi) were analyzed by northern blot hybridization or RT-PCR, and real-time PCR. The accumulation of both *OsMADS7* and

Table 1 Characteristics of the florets in transgenic lines

Plants	Palea[a]/lemma[b]	Lodicules[c]/No. of lodicules	Stamens[d]/No. of stamens	Carpels[e]/No. of styles	Extra whorls[f]
Wild type	–/–	–/2(100%)	–/6(100%)	–/2(100%)	–/grains
OsM1/*5*/*7*/*8*-RNAi_6	++/+	+++	+++	+++	+++
OsM1/*5*/*7*/*8*-RNAi_9	++/+	+++	+++	+++	+++
OsM7/*8*-RNAi_2	–/–	++/2(100%)	+/6(94%), 5(6%)	+ (59%), ++ (41%)	++
OsM7/*8*-RNAi_5	–/–	++/2(85%), 3(10%), 4(5%)	+/6(68%), 5(32%)	+ (55%), ++ (45%)	++
OsM7/*8*-RNAi_8	–/–	+/2(89%), –/2(11%)	+/6(50%), 5(22%), 4(28%)	+ (9%), ++ (91%)	++
OsM7/*8*-RNAi_14	–/–	++/2(85%), 3(15%)	+/6(63%), 5(37%)	+ (24%), ++ (76%)	++
OsM8-RNAi_1	–/–	–/2(100%)	–/6(100%)	–/2(100%)	–/grains
OsM8-RNAi_4	–/–	–/2(100%)	–/6(100%)	–/2(100%)	–/grains
OsM7-RNAi_3	–/–	–/2(100%)	–/6(100%)	–/2(60%), 3(20%), 4(20%)	–/grains[g]
OsM7-RNAi_6	–/–	–/2(100%)	–/6(100%)	–/2(100%)	–/grains[g]

[a]normal; ++, paleas were leaf-like.
[b]normal; +, lemmas were longer and narrower than the WT.
[c]normal; +, lodicules were slender and papery; ++, lodicules were transformed into palea/lemma-like structures; +++, lodicules were leaf-like.
[d]normal; +, stamens were longer and thinner than those of WT; +++, stamens were absent or transformed into leaf-like structures.
[e]normal; +, carpels were green and palea/lemma-like structures fused partially to form 2–4 abnormal styles; ++, carpels were broader than in the WT and not fused, the stigmas and styles were not visible, some with multi-carpel structures; +++, carpels were transformed into leaf-like structures.
[f]–/grains, normal and finally seed can be got; +, abnormal organ-like structures inside the carpels with mild phenotypes; ++, stamen- or carpel-like organs developed from the center of the carpels with strong phenotypes; +++, abnormal panicles with an elongated pedicel in the center of the florets.
[g]The fertility was affected.
Note: More than 200 spikelets were dissected from each of the transgenic lines.

OsMADS8 transcripts were strongly decreased, while the expression of *OsMADS1*, *OsMADS5* and *OsMADS34* was upregulated compared with the wild type (Figs 1b and S2). Four of the transgenic lines were analysed further in detail (Table 1).

Wild-type plants were flowering at about 73 DAP (days after planting), whereas all the transgenic lines were delayed in heading (Fig. S3) by approximately 2 weeks, with flowering ranging from 84 to 92 DAP. However, the transition stage from shoot meristem to inflorescence meristem was not delayed. The glumes in the spikelets of transgenic plants developed normally, and also palea and lemma largely resembled that of the wild type (Fig. 2a,c). The most significant morphological changes in *OsM7/8*-RNAi knockdown lines were observed in the organs of the innermost three whorls (Table 1). The lodicules were transformed into lemma/palea-like structures, some of which appeared to be papery and thin (Fig. 2e,f), arrowheads); the number of these organs was sometimes increased and varied from two to four. The anthers and filaments of stamens of the knockdown lines appeared to be longer and thinner (Fig. 2h, arrows) than those of the wild type. The number of stamens in florets varied from three to seven. However, no pollen grains were produced by these stamens (Table 1).

In *OsM7/8*-RNAi knockdown plants all carpels were aberrant, but showed a range of phenotypes. In mild cases carpels were green and partially fused (Fig. 2f,g). In plants with the strongest phenotypes the carpels were completely unfused (Fig. 2i, black arrows). In addition, the determinacy of the flower meristems in *OsM7/8*-RNAi knockdown plants was lost, so that inside the carpels additional reproductive organ-like structures were initiated (Fig. 2g,i, white arrows). The alterations of the modified florets were inspected more closely by scanning electron microscopy (SEM). In *OsM7/8*-RNAi knockdown plants, the abaxial epidermal cells of the lodicules, stamens and carpels were changed in size and shape (Fig. 2l,n,p), compared with those of the wild type (Fig. 2k,m,o). Remarkably, trichome-like structures emerged on the abaxial epidermal surface of the transgenic lodicules (Fig. 2h, arrowhead), stamens (Fig. 2n, arrow) and carpels (Fig. 2p, arrow), a situation similar to that of wild-type palea and lemma (Fig. 2j), suggesting that stamen and carpel identities were transformed homeotically into palea/lemma-like organ identity, at least partially.

Taken together, *OsM7/8*-RNAi knockdown plants showed both severe meristic and homeotic effects in the inner three floral whorls, while the other floret and spikelet organs appeared much less affected, if modified at all.

Simultaneous silencing of *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8* transforms all floret organs into leaf-like structures

In order to uncover potential functional redundancy beyond *OsMADS7* and *OsMADS8* rice lines transgenic for the pUJOsM8M construct were generated in which four *SEP*-like genes, *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8* are knocked down. 26 independent transgenic lines showed severe phenotypes. Two independent lines (*OsM1*/*5*/*7*/*8*-RNAi_6 and *OsM1*/*5*/*7*/*8*-RNAi_9) in which the expression of *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8*, but not

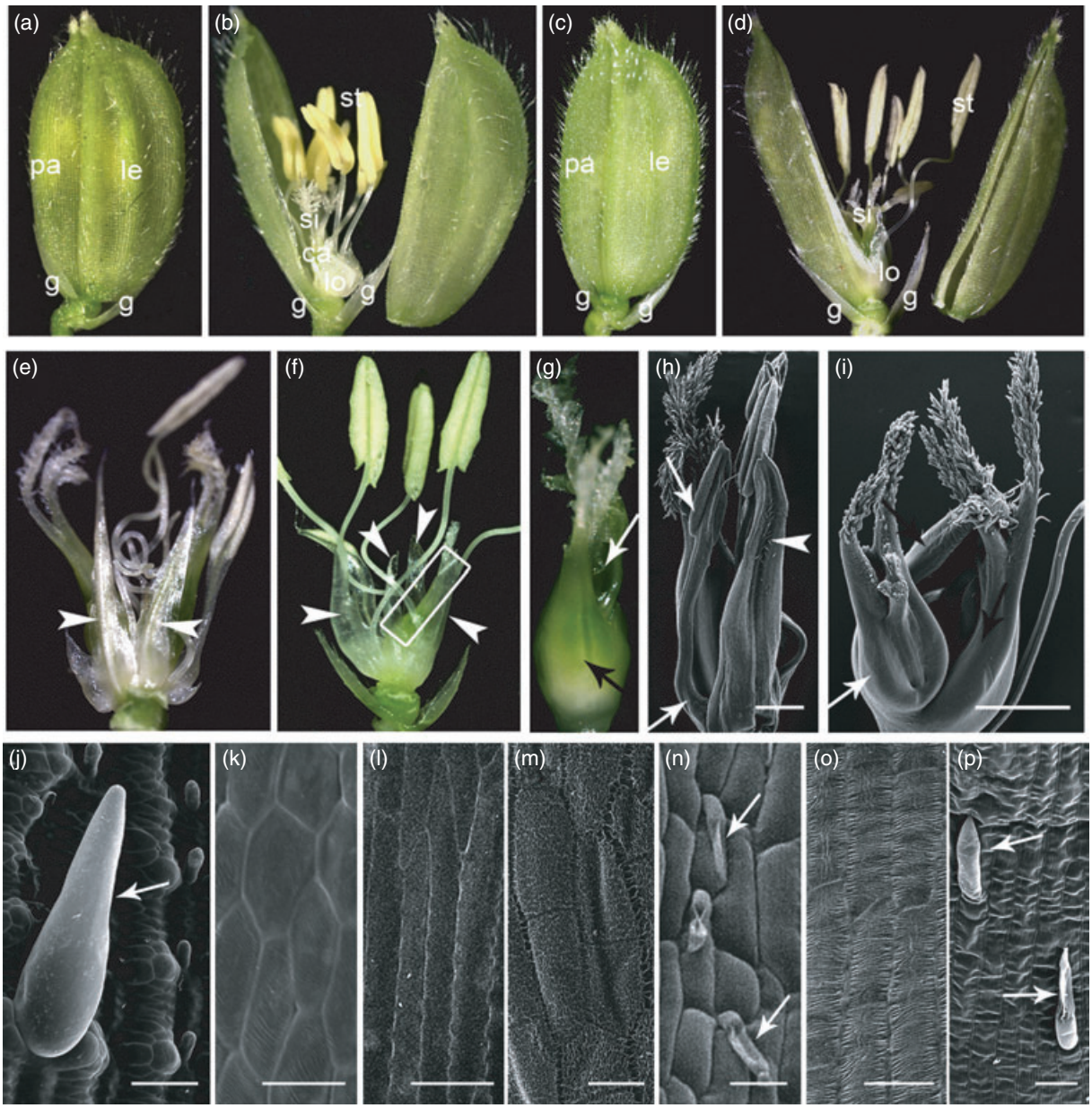

Figure 2. Phenotypes of wild-type and *OsM7/8*-RNAi spikelets.
(a) A wild-type spikelet with normal glumes, palea and lemma.
(b) A wild-type floret with normal lodicules, stamens, carpel and styles after lemma and palea were opened.
(c) The *OsM7/8*-RNAi transgenic spikelet with normal glumes, palea and lemma.
(d) The *OsM7/8*-RNAi transgenic spikelet showing that the floral organs of the inner three whorls are affected.
(e, f) The *OsM7/8*-RNAi transgenic spikelet showing the homeotic transformation of lodicules into lemma/palea-like organs in whorl 2 (arrowheads). For clarity, palea and lemma have been removed.
(g) Close-up of the carpel in (f). Black arrow indicates carpel and white arrow indicates additional carpel.
(h) SEM of the *OsM7/8*-RNAi aberrant stamens and lodicules. Arrows indicate the flat and thin anther and filament, and the arrowhead indicates the lodicules. Bar = 500 μm.
(i) SEM of the *OsM7/8*-RNAi aberrant carpel. Black arrow indicates carpel and white arrow indicates additional carpel. Bar = 500 μm.
(j) SEM of abaxial epidermal surface of wild-type lemma with trichomes. Bar = 30 μm.
(k) SEM of abaxial epidermal surface of wild-type lodicules.
(l) SEM of abaxial epidermal surface of the *OsM7/8*-RNAi lodicules.
(m) SEM of abaxial epidermal surface of wild-type anthers.
(n) SEM of abaxial epidermal surface of the *OsM7/8*-RNAi anther-like structures. Bar = 20 μm in (k–n).
(o) SEM of abaxial epidermal surface of wild-type carpels.
(p) SEM of abaxial epidermal surface of the *OsM7/8*-RNAi carpels. Bar = 15 μm in (o, p). Arrows in (j, n, p) indicate trichome and trichome-like structures. g, glume; ca, carpel; le, lemma; lo, lodicule; pa, palea; si, styles; st, stamen.

Figure 3. Phenotypes of the *OsM1*/*5*/*7*/*8*-RNAi inflorescences and spikelets.
(a) Comparison of the wild-type (left) and *OsM1*/*5*/*7*/*8*-RNAi_6 (middle), *OsM1*/*5*/*7*/*8*-RNAi_9 (right) panicle.
(b) The *OsM1*/*5*/*7*/*8*-RNAi spikelet shows that the palea was affected more severely than the lemma.
(c) The *OsM1*/*5*/*7*/*8*-RNAi spikelet showing that the paleas and all floret organs became leaf-like. Additional inflorescence-like structures (arrow) developed in whorl 4 (w4).
(d) The *OsM1*/*5*/*7*/*8*-RNAi spikelet showing the malformed extra spikelet (arrowheads).
(e, f) *OsM1*/*5*/*7*/*8*-RNAi florets of transgenic plants with strongest phenotypes revealing that all organs of the three innermost whorls are homeotically transformed into leaf-like structures. The arrow indicates the additional abnormal inflorescence that developed from an extra whorl within whorl 4. For clarity, palea and lemma have been removed in (d–f).
(g, h) SEM of different developmental stages of the *OsM1*/*5*/*7*/*8*-RNAi carpels. Arrows indicate the young inflorescence-like structures emerging inside the carpel. Bars = 50 μm in (g, h).
(i) SEM of a malformed extra spikelet that emerges in the additional inflorescence-like structures at later developmental stages from the center of the *OsM1*/*5*/*7*/*8*-RNAi carpel. Bar = 100 μm.
(j) SEM of abaxial epidermal surface of the *OsM1*/*5*/*7*/*8*-RNAi carpel. The arrow indicates a hair-like structure on the surface. Bar = 10 μm. ca, carpel; g, glume; le, lemma; lo, lodicule; pa, palea; w2, whorl 2; w3, whorl 3; w4, whorl 4.

of *OsMADS34*, were strongly down-regulated (Fig. 1c), were selected for further analysis (Table 1). As revealed by RT-PCR and real-time PCR the expression of other floral homeotic genes or their close relatives (e.g. *OsMADS14*, *OsMADS15*, *OsMADS4*, *OsMADS16*, *OsMADS3*, and *OsMADS58*) in the two lines was not or only weakly affected (Fig. S4a,b).

The *OsM1*/*5*/*7*/*8*-RNAi knockdown lines showed mutant phenotypes very similar to those of the strong *osmads1* mutant (Agrawal *et al.*, 2005) and the *dsRNAiOsM1* lines (Prasad *et al.*, 2005), including abnormal panicles (Fig. 3a), under-developed palea and lemma (Fig. 3b,c), and sterility (Table 1). However, there were also interesting differences. The *OsM1*/*5*/*7*/*8*-RNAi knockdown lines were tremendously delayed by almost 3–4 weeks in their flowering time (Fig. S3). In a few plants with the strongest phenotype the panicles did not appear out of flag-leaves. Moreover, the palea, but not the lemma, were more severely affected in the *OsM1*/*5*/*7*/*8*-RNAi knockdown transgenic lines (Fig. 3 b,c) compared with the *osmads1* mutant (Agrawal *et al.*, 2005) and the *dsRNAiOsM1* lines (Prasad *et al.*, 2005). All lodicules, stamens and carpels were homeotically transformed into leaf-like structures (Fig. 3c–f). The additional abnormal panicles with an elongated pedicel in the center of the floret (Fig. 3c,d,f) developed from the extra whorl, or simultaneously in the third whorl (Fig. 3d). The different developmental stages of the additional abnormal panicles were inspected by scanning electron microscopy (SEM). Inflorescence-like structures were developed from inside the carpels (Fig. 3g,h) at both early and late developmental stages (Fig. 3i). SEM also showed that hair-like structures are present on the surfaces of the organs that have been homeotically transformed into leaf-like structures in the fourth whorl position (Fig. 3j). These phenotypic features resemble greatly those of the *sep1*/*2*/*3*/*4* quadruple mutant of *Arabidopsis* in which all floral organs are transformed into leaf-like structures and the flower lost determinate growth.

To confirm that the severely abnormal phenotypes of the *OsM1*/*5*/*7*/*8*-RNAi transgenic plants are the result of RNAi, the presence of *OsMADS8*-derived small (21–24 nt) RNAs was investigated by Northern blot hybridization. Our data showed that siRNAs were detectable from the transgenic lines with abnormal phenotypes, but not from control plants transformed with the empty vector (Fig. S5b), corroborating the view that the abnormal phenotypes were indeed caused by RNAi.

Comparison of expression patterns of *OsMADS7* and *OsMADS8*

Previously, northern blot analysis showed that the expression of *OsMADS7* and *OsMADS8* is restricted to reproductive organs such as inflorescences, and developing kernels (Pelucchi *et al.*, 2002). *In situ* hybridization showed that during flower development both genes are expressed in the part of the floral meristem where the lodicule and stamen primordia will originate. Later on expression is found in lodicules, developing stamens and pistils until flowers mature (Pelucchi *et al.*, 2002). To better understand the mutant phenotypes described above with respect to gene expression the temporal and spatial expression patterns of the paralogous genes were analyzed by *in situ* hybridization in more detail involving a series of panicle and spikelet developmental stages. To facilitate the description of the observed expression patterns, the designation of developmental stages proposed previously was used (Itoh *et al.*, 2005).

At very early stages of inflorescence development no *OsMADS7* signal was visible (Fig. 4a,b); meanwhile the *OsMADS8* transcripts were first detected in the primary branch meristems and at the tip of the bracts (bract 1 and 2) (Fig. 4c,d). Subsequently, an *OsMADS7* signal became visible but was very restricted to the spikelet meristems (Fig. 4e,f), while *OsMADS8* was expressed strongly and broadly both in the branch shoots and spikelet meristems (Fig. 4g). The expression domains of *OsMADS8* and *OsMADS7* are overlapping during spikelet development (Fig. 4h–m), but appear to be slightly different spatially during the early development of floral meristems (Fig. 4h,i). Subsequently, the transcripts of *OsMADS8* and *OsMADS7* were localized in the developing lodicules, stamens and pistils (Fig. 4j,k). In the mature florets, the transcripts of both *OsMADS8* and *OsMADS7* were confined to the reproductive organs (i.e. the stamens and ovary) (Fig. 4l,m).

Interaction patterns among rice SEP-like proteins

Previous studies showed that the OsMADS7 and OsMADS8 proteins are able to interact with candidate class A, class B and class C floral organ identity proteins, a situation similar to that of the SEP proteins in *Arabidopsis*, whereas OsMADS1 only interacts with putative class A (AP1-like) proteins (Moon *et al.*, 1999; Lim *et al.*, 2000). We investigated the interaction between all the rice SEP proteins under study in this manuscript *in vitro* and *in vivo*. Yeast two-hybrid assays showed that OsMADS7, OsMADS8 and OsMADS1 share similar interaction patterns at different strengths. As shown in Figure 5, both OsMADS7 and OsMADS8 are able to form homodimers (Fig. 5k,p), while OsMADS1 can homodimerize only weakly (Fig. 5a). These proteins also interact with each other (Fig. 5c,d,l,n,q,s). However, OsMADS5 can neither homodimerize, nor heterodimerize with the other SEP proteins (Fig. 5b,f,g,h,l,r). In addition, we analysed the interaction of rice class E proteins in *Arabidopsis* mesophyll protoplasts. Coimmunoprecipitation assays showed that OsMADS7 and OsMADS7, OsMADS7 and OsMADS1, OsMADS7 and OsMADS8, OsMADS8 and OsMADS8, OsMADS8 and OsMADS1, and OsMADS1 and OsMADS1 undergo protein–protein interactions in *Arabidopsis* mesophyll protoplasts, while OsMADS5

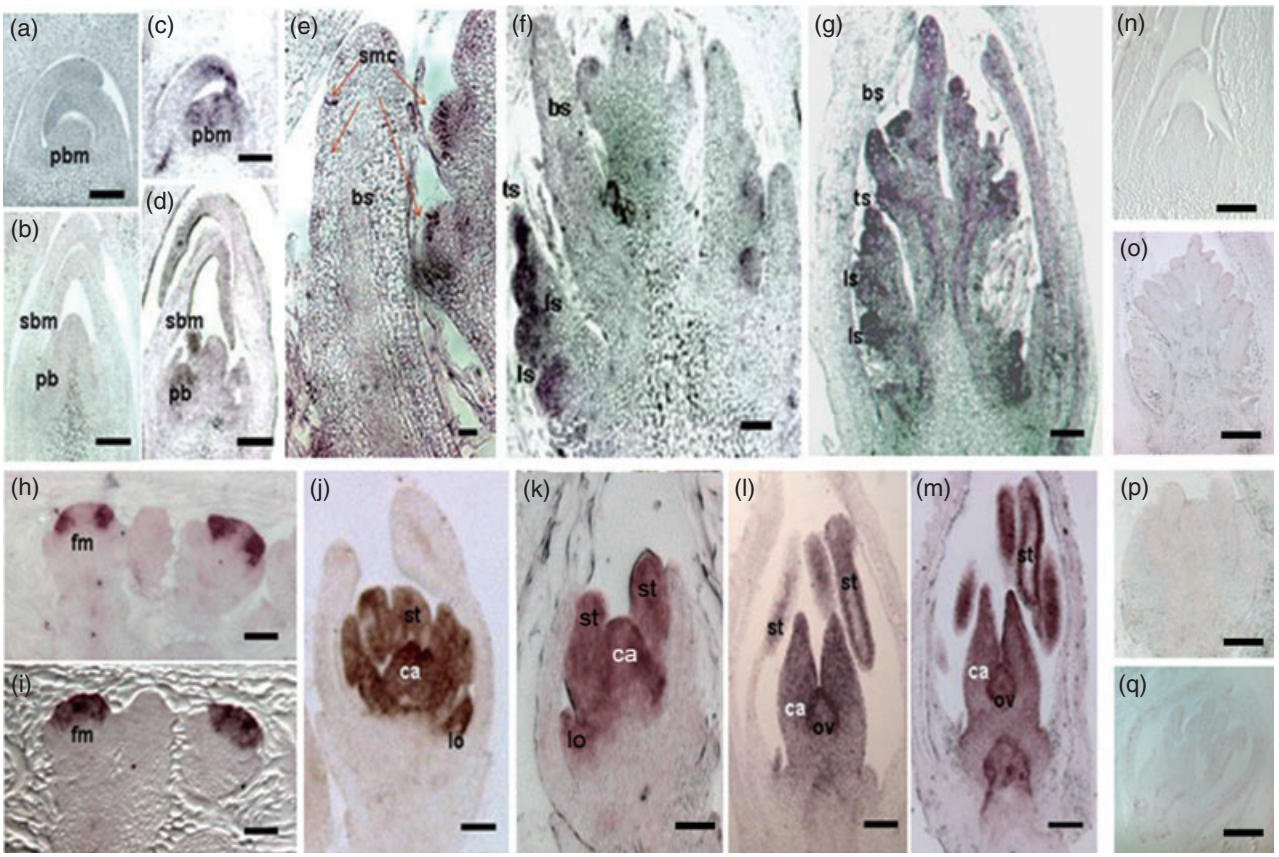

Figure 4. Expression patterns of *OsMADS7* and *OsMADS8* during inflorescence and spikelet development as revealed by *in situ* hybridization.
(a–m) Anti-sense probe hybridization for *OsMADS7* and *OsMADS8*.
(a, b) Transcripts of *OsMADS7* were not detectable in primary branch meristems (pbm) and secondary branch meristems (sbm).
(c, d) Transcripts of *OsMADS8* were first detected during the initiation of primary branch meristems (pbm) and secondary branch meristems (sbm).
(e, f) The *OsMADS7* signal is restricted to the spikelet meristems, the terminal spikelet meristem (tsm) and lateral spikelet meristem (lsm). In (e), close-up of the branch shoot (bs) showing that the signal is restricted to the spikelet meristem cells (smc) (arrows).
(g) *OsMADS8* is expressed strongly and broadly both in the branch shoots and spikelet meristems.
(h) The *OsMADS7* signal is restricted to the floral meristem primordia (fm) of whorl 3.
(i) *OsMADS8* expression is visible in the floral meristem primordia (fm) of whorls 3 and 4.
(j, k) The *OsMADS7* in (J) and *OsMADS8* in (K) transcripts are detected in the developing lodicule (lo), stamen (st) and carpel (ca).
The *OsMADS7* in (l) and *OsMADS8* in (m) signals are visible in the reproductive organs (stamens and carpels including ovules) in mature florets.
(n–q) Negative control of sense probe for *OsMADS8* in (n, o), and *OsMADS7* in (p, q). Bar = 0.5 mm.

does not interact with OsMADS1, OsMADS7 or OsMADS8 (Fig. 6).

DISCUSSION

OsMADS7 and *OsMADS8* have a class E floral homeotic gene function in rice

In *Arabidopsis*, *SEP1*, *2* and *4* are expressed throughout the floral meristems already at developmental stage 2, while *SEP3* expression starts a bit later in the region in which floral organ primordia will initiate soon; subsequently, expression of *SEP1* and *2* is restricted to floral organs of all four whorls and *SEP3* expression to the inner three whorls, whereas *SEP4* expression is localized in the central dome and only weakly in sepals (Flanagan and Ma, 1994; Mandel and Yanofsky, 1998). This finding, together with their considerable sequence similarity, suggests that the *SEP* genes encode proteins with redundant functions. This situation is indeed the case, as single mutants for each of the *SEP* genes generated by reverse genetics display only very weak mutant phenotypes, if any, while triple and quadruple mutants show homeotic transformations of floral organs and a dramatic loss of floral determinacy (Pelaz *et al.*, 2000, 2001; Ditta *et al.*, 2004).

In rice, the expression patterns of *SEP*–like genes are quite heterogeneous. *OsMADS1* is first expressed in the spikelet meristem before the glume primordia emerge, and then restricted to lemma and palea, with weak expression in the carpel (Chung *et al.*, 1994; Prasad *et al.*, 2001). Expression of *OsMADS5* was detected in primordia of stamens and (weakly) carpels, but not in those of lemma and palea (Kang and An, 1997). *OsMADS34* is expressed

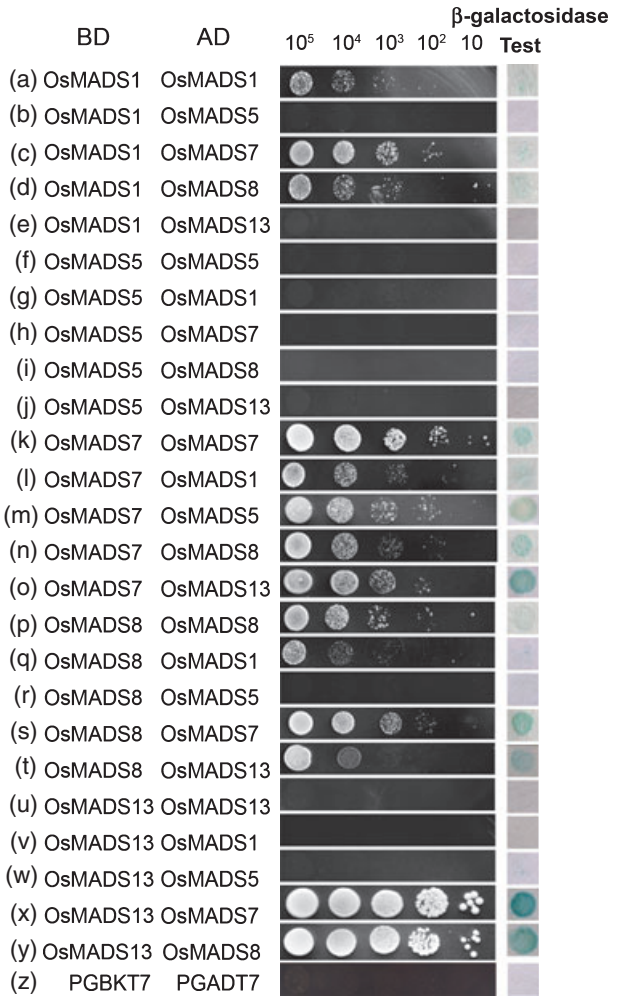

Figure 5. Yeast two-hybrid assays. Interaction patterns of the rice SEP-like proteins including OsMADS1, OsMADS5, OsMADS7 and OsMADS8 are shown.
Serial dilutions of 10^5–10^1 AH109 cells containing different plasmid combinations were grown on the selective medium SD-LTHA + 5 mM 3-AT. L, leucine; T, tryptophan; H, histidine; A, adenine; 3-AT, 3-amino-1,2,4-triazole. Dilution of 10^4 stained for β-galactosidase activity.

throughout the plant, but expression is not detectable in the organs of the second and fourth whorls at later stages of flower development (Pelucchi *et al.*, 2002). The expression patterns of these *SEP*–like genes in rice make them no obvious candidates for being the only class E genes in a strict sense (Becker and Theissen, 2003), but do not exclude subfunctionalization of class E genes. Expression of *OsMADS8* and *OsMADS7* was first detected in spikelet meristems, and then not detected in lemma and palea primordia, but transcripts of both genes were found to accumulate in developing lodicules, stamens and carpels during spikelet development (Fig. 4) (Greco *et al.*, 1997). The expression patterns of these two genes are thus quite similar to those of *SEP1*, *SEP2*, and *SEP3* in *Arabidopsis*, suggesting that *OsMADS8* and *OsMADS7* play a corresponding role in rice.

Previously, data on gene function of grass *SEP*–like genes based on mutant phenotypes were only available for *OsMADS1* and *OsMADS5*. Some missense mutations in *OsMADS1* result in the *lhs1* mutant phenotype, suggesting that *OsMADS1* plays a role in specifying meristem, palea and lemma identities (Jeon *et al.*, 2000). More recently it was shown that knockdown of *OsMADS1* affects the differentiation of specific cell types in lemma and palea, thus generating glume-like features, with severe derangements in the lemma (Prasad *et al.*, 2005). In contrast, ectopic expression of *OsMADS1* causes conversion of the glumes into lemma-/palea-like organs, but does not result in morphological alteration of floral organs (Prasad *et al.*, 2001, 2005). The organs of the inner whorls in many *OsMADS1* knockdown florets are converted into glume-like organs (Prasad *et al.*, 2005; Chen *et al.*, 2006).

Severe loss-of-function mutants affected in *OsMADS1* display complete homeotic transformations of the organs of the three inner whorls (lodicules, stamens and carpels) into lemma- and palea-like structures, and a loss of determinacy of the flower meristem (Agrawal *et al.*, 2005). If one equates palea/lemma with sepals, this

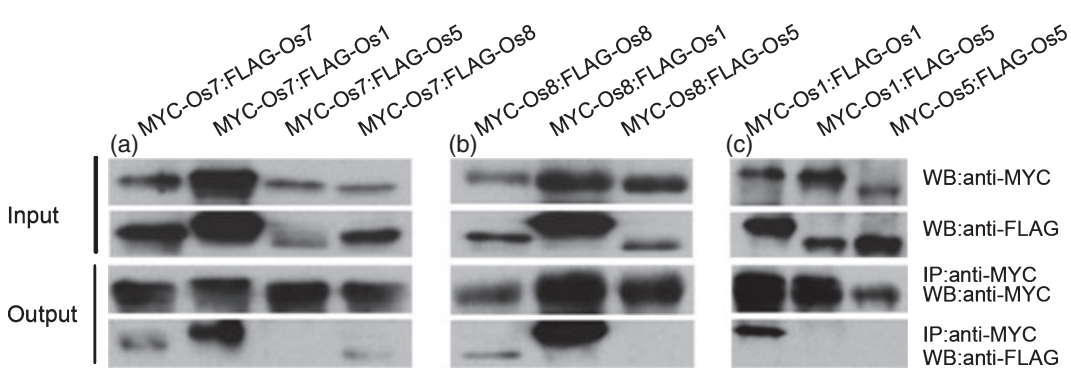

Figure 6. Interaction analyses of four rice class E proteins *in vivo*.
(a) Co-IP of OsMADS7 and OsMADS7/1/5/8 proteins.
(b) Co-IP of OsMADS8 and OsMADS8/1/5 proteins.
(c) Co-IP of proteins OsMADS1 and OsMADS1/5, and OsMADS5 and OsMADS5.

phenotype strikingly resembles that of the *sep1/2/3* triple mutant of *Arabidopsis* (Pelaz *et al.*, 2000), which led to the definition of the class E floral homeotic function (Theissen, 2001). Such a function may appear difficult to reconcile with the fact that *OsMADS1* is not expressed in lodicules and stamens, which might be explained by very early effects of *OsMADS1*, when the gene is expressed in the spikelet primordium before organ primordia develop. A function of *OsMADS1* at this stage may be required for the specification of floral organs at later stages of ontogeny (Prasad *et al.*, 2005).

In case of the loss-of-function of *OsMADS5*, the closest relative of *OsMADS1* in the rice genome, the only deviation from wild-type phenotype found in the whole plant was that lodicules were attached to the lemma and palea, suggesting that the gene is not required for class E function (Agrawal *et al.*, 2005), even though it may contribute to it in a redundant way.

Like in case of *osmads1* mutants, the morphogenetic alterations in our studies, caused by knockdown of both *OsMADS7* and *OsMADS8*, were also restricted to the organs of the innermost three whorls. Specifically, the lodicules, stamens and carpels were transformed into palea/lemma-like structures, whereas glumes, lemma and palea were not affected. Flower apical meristems (AMs) in the center of the transgenic carpels were reverted into reproductive meristems, which developed into stamen-like structures or carpel-like structures. As either of the single gene knockdowns does not cause the morphological change, our results strongly suggest that *OsMADS7* and *OsMADS8* are functionally redundant, but that normal function of at least one of these genes is required for proper development of organ identities in the inner three floral whorls, and for maintenance of flower meristem determinacy. Furthermore, the knockdown *OsMADS7/8* transgenic plants were delayed in flowering time by approximately 2 weeks, but not in the transition from shoot meristem to inflorescence meristem, thus resembling *sep1/2/3* loss-of-function mutants in *Arabidopsis* (Jack, 2001; Theissen and Saedler, 2001). Considering that *OsMADS7/8* is expressed at every developmental stage from branch meristems to floral organs, we hypothesize that late heading caused by loss-of-function of *OsMADS7/8* may be due to the affected stem elongation and bolting.

The lack of redundancy to other *SEP*-like genes may explain why at least two 'natural' mutants, *leafy hull sterile1* (*lhs1*) and *naked seed rice* (*nsr*), have been found for *OsMADS1*. This is in contrast to the redundantly acting *OsMADS7* and *OsMADS8* and the *SEPALLATA* genes of *Arabidopsis*, which required the generation of double or even triple mutants obtained by reverse genetics to display a mutant phenotype. The mutant phenotypes of *osmads1* and *osmads7/8* are strikingly similar, indicating that these genes do not act redundantly such as *SEP1/2/3*, but in an interdependent way.

Evolutionary implications of class E genes in rice

Phylogenetic analyses revealed that *SEP*-like genes can be further subdivided into several well supported clades (subfamilies), and that many duplications of *SEP*-like genes occurred during angiosperm evolution (Malcomber and Kellogg, 2005; Zahn *et al.*, 2005). For each of the five rice *SEP*-like genes, there are putative orthologs identified from other grass species, such as maize (Münster *et al.*, 2002) and ryegrass (Petersen *et al.*, 2004), indicating that the most recent common ancestor of maize and rice, which existed quite close to the base of extant grasses about 50–70 million years ago, had already at least five different *SEP*-like genes (Münster *et al.*, 2002; Becker and Theissen, 2003). A recent phylogenetic analysis provided evidence for an ancient duplication of *SEP*-like genes before the origin of the most recent common ancestor of extant angiosperms that generated two subclades, *SEP1/2/4*-like genes (or *AGL2/3/4* clade) and *SEP3*-like genes (*AGL9* clade), with *OsMADS8* and *OsMADS7* being *SEP3*-like genes, and *OsMADS1*, *OsMADS5* and *OsMADS34* being *SEP1/2/4*-like genes (Zahn *et al.*, 2005). The expression patterns of *SEP1/2/4*-like genes vary considerably among cereals, suggesting that these genes evolved diverse functions which may have facilitated the origin of diverse inflorescence structures in grasses (Malcomber and Kellogg, 2004; Shitsukawa *et al.*, 2007).

Our results provide strong evidence that an ancestral function of class E floral homeotic genes in being involved in specifying the identity of all kinds of floral organs is conserved through the angiosperms, although the functional partitions among the paralogs vary in different species. While the situation in *Arabidopsis* is largely dominated by functional redundancy among the *SEP* genes (Pelaz *et al.*, 2000; Ditta *et al.*, 2004), a whorl-specific subfunctionalization was observed in another eudicot, *Gerbera* (Uimari *et al.*, 2004). In the monocot rice, however, the functional interdependence of two types of genes, *OsMADS1* and *OsMADS7/8*, evolved, a situation that can somewhat be considered being the opposite of functional redundancy. The fact that *osmads1* and *osmads7/8* mutants have similar, but not identical functions, such as in the control of flowering time, could indicate that individual proteins have additional functions beyond their activities in complexes involving both types of proteins.

The obligate functional interdependence of floral homeotic genes, as observed here for *OsMADS1* and *OsMADS7/8*, is not unprecedented, but well-known from the class B floral homeotic genes of eudicots. In these cases the formation of obligate heterodimers between DEF- and GLO-like proteins upregulating the expression of the *DEF*-like and *GLO*-like genes in an autoregulatory loop explains why both genes are functionally interdependent, so that mutants in the *DEF*-like gene are almost identical to mutants in the *GLO*-like gene (Schwarz-Sommer *et al.*,

1992; Lenser *et al.*, 2009). This system possibly evolved to increase the robustness of important decisions during the development of flower organ identity (Lenser *et al.*, 2009). A similar explanation may apply to the *SEP*-like genes of rice. Here, the class E gene function specifying the identity of lodicules, stamens and carpels may require the direct interaction of OsMADS1 and OsMADS7/8 during spikelet meristem development, when the expression patterns of these genes overlap. For example, protein complexes involving OsMADS1–OsMADS7 dimers may act redundantly with those involving OsMADS1–OsMADS8 dimers in specifying lodicules, stamen and carpel identity, with OsMADS1 being required in both complexes. In contrast, complexes involving OsMADS7 or OsMADS8 but not OsMADS1 may have a specific role in determining heading date or palea development.

However, there is a clear difference between the functional interdependence of *DEF*-like and *GLO*-like class B floral organ identity genes and the case discussed here. The interdependent positive autoregulatory loops in which *DEF*-like and *GLO*-like genes are involved imply that expression of the one gene is almost abolished when the function of the other gene is compromised, and *vice versa* (Schwarz-Sommer *et al.*, 1992; Lenser *et al.*, 2009). However, knockdown of *OsMADS7*/*8* expression does not reduce but upregulates *OsMADS1* expression. Thus the remaining activity of OsMADS7/8 or OsMADS1, respectively, either suffices to sustain the autoregulatory loop, or the respective genes do not exhibit a regulatory relationship in such a way at all. The upregulation seen may even suggest that the genes are connected by negative rather than positive feedback loops, either directly or indirectly. It is becoming more and more clear that many MADS-box genes are subject to positive or negative cross- and autoregulatory control (see, e.g., Gómez-Mena *et al.*, 2005; Lenser *et al.*, 2009; Liu *et al.*, 2009; Ohmori *et al.*, 2009), so that such a scenario may not appear too far fetched.

Alternatively, the similarity of OsMADS1 and OsMADS7/8 loss-of-function phenotypes may reflect a dosage effect during spikelet meristem development. Assuming that the genes are functionally almost equivalent, but dosage dependent (incomplete) knock-down of either OsMADS7 or OsMADS8 may still leave sufficient protein, while knockdown of both genes, or of OsMADS1, may bring the OsMADS1/7/8 amount in the spikelet meristem cells below a critical threshold for proper SEP-like protein function. Both *osmads1* and *osmads7/8* mutants reveal some aspects of the severe and complete *sep* phenotype when the four *SEP*-like genes (*OsMADS1*/*5*/*7*/*8*) are down-regulated, suggesting that these class E paralogs have undergone subfunctionalization resulting in partial overlapping functions.

It may be worthwhile to note that *OsMADS34*, also a *SEP*-like gene in rice that is expressed in all plant tissues, was not negatively affected in its expression in all the transgenic rice lines studied by us, as revealed by real-time PCR and RT-PCR. Remarkably, its expression level in the transgenic lines was even higher than that in wild-type plants, suggesting that the transcription of *OsMADS34* may be usually repressed by the other *SEP*-like genes, either directly or indirectly. However, our data show that the upregulation of *OsMADS34* is insufficient to compensate for the decreased expression of the other *SEP*-like genes.

As shown above, *OsM1*/*5*/*7*/*8*-RNAi knockdown lines resemble greatly those of the *sep1*/*2*/*3*/*4* quadruple mutant of *Arabidopsis*. This finding suggests that genes *OsMADS1*/*5*/*7*/*8* cover the full class E floral homeotic function, even though we cannot completely exclude the possibility that a quintuple mutant also comprising *OsMADS34* would not reveal an even more severe class E loss-of-function phenotype.

As summarized in the 'floral quartet model,' the SEP proteins form higher order complexes together with class A, B or C proteins to control various transcriptional programs required and sufficient for specifying floral organ identity during development in eudicots (Theissen, 2001; Theissen and Saedler, 2001). Our data show that some phenotypes caused by silencing of the *SEP* orthologs (*OsMADS7* and *OsMADS8*) resemble those of the class B (*OsMADS16*) or class C (*OsMADS3* and *OsMADS58*) gene mutants (Nagasawa *et al.*, 2003). Taking together, these data suggest that 'floral quartet' complexes, similar to those in eudicot species (Honma and Goto, 2001; Favaro *et al.*, 2003), may also be formed to control flower development in rice. More data on protein–protein and protein–DNA interactions, as provided, e.g. by electrophoretic mobility shift assays and yeast two-hybrid assays (Immink *et al.,* 2009; Melzer and Theissen, 2009, Melzer *et al.,* 2009; this study) are required to clarify the issue.

Previously, comparison of loss-of-function mutants of class B genes in maize (Ambrose *et al.*, 2000; Whipple *et al.*, 2004) and rice (Nagasawa *et al.*, 2003) with those in eudicots have shown that the lodicules of grasses are homologous to eudicot petals. A recent study provided evidence that class B genes have a conserved function involved in specifying second whorl organ identity across the angiosperms (Whipple *et al.*, 2007). Our findings that lodicules are transformed into palea/lemma-like organs after knockdown of *OsMADS7*/*8* in transgenic plants, and that lodicules are transformed into leaf-like structures after silencing of *OsMADS1*/*5*/*7*/*8* in transgenic plants provide further evidence that there is a common conserved mechanism for specification of second whorl organ identity throughout flowering plants.

EXPERIMENTAL PROCEDURES

PLANT MATERIALS

Plants of *O. sativa* ssp. *Japonica* variety 'Zhonghua 11' were grown in local paddy-fields and the greenhouse of the Institute of Botany,

Chinese Academy of Sciences, Beijing (latitude of 39̊48′ N and a longitude of 116̊28′ E).

Generation of knockdown vectors for rice *SEP*-like genes

Specific regions of either *OsMADS7* or *OsMADS8* used for the RNAi constructs (Fig. S5a) were defined by the alignment of the rice candidate floral homeotic genes (Table S1). Cloning of the *OsM7C* region in sense and antisense orientation yielded vector pUJOsM7C, predicted to specifically knockdown *OsMADS7*; likewise, pUJOsM8C containing the *OsM8C* region was expected to knockdown *OsMADS8*, pUJOsM7I to knockdown both *OsMADS7* and *OsMADS8*, and pUJOsM8M to simultaneously knockdown *OsMADS1*, *OsMADS5*, *OsMADS7* and *OsMADS8* by RNAi in transgenic rice plants. The intron and nos terminator cassette of pJawohl3–RNAi (GenBank Accession no. AF404854) were transferred with *Bam*HI/*Not*I sites to the pBluescript SK (Stratagene, La Jolla, CA, USA), termed pBJWI3 as an intermediate vector. Then the desired coding regions (*OsM7C*, *OsM8C*, *OsM7I*, *OsM8M* in Fig. S5a) were amplified with specific primers (Table S2) and cloned into two sides of the intron region of the pBJWI3 vector in the sense and antisense orientation, respectively. Finally, the dsRNAi cassette containing the intron and two oppositely orientated coding sequences were mobilized with restriction enzymes *Bam*HI and *Sac*I and introduced into vector pCAMBIA1301–Ubi, in which the maize (*Zea mays*) *Ubi* (ubiquitin) promoter (Cornejo *et al.*, 1993) was inserted into the *Hin*dIII and *Bam*HI sites, thus resulting in final plasmids pUJOsM7C, pUJOsM8C, pUJOsM7I and pUJOsM8M. All these constructs were verified by restriction mapping.

Rice transformation

Rice calli, which had been induced from mature embryos of variety Zhonghua11 (*O. sativa* ssp. *Japonica*), were transformed by the *Agrobacterium* strain EHA105 harboring one of the RNAi constructs or the control vector pCAMBIA1301, as described previously (Hiei *et al.*, 1994; Huang *et al.*, 2000). Transgenic calli were selected on Murashige and Skoog (MS) medium containing 50 mg L^{-1} hygromycin B (Roche, cat.No.10843555001). Hygromycin-resistant plants regenerated from calli were transplanted into soil and grown at a greenhouse or local paddy-fields. For measurement of flowering time, the transgenic and wild-type plants were transplanted under natural short day conditions (in paddy-fields in Beijing).

Scanning electron microscopy

Inflorescences from transgenic or wild-type rice were fixed in fresh FAA solution (50% ethanol, 5% acetic acid, and 3.7% formaldehyde), dried, coated as described (Shan *et al.*, 2006), and photographed with a Hitachi S-800 scanning electron microscope (SEM).

In situ hybridization

Freshly collected young panicles were fixed immediately in FAA solution. The gene-specific C-terminal regions of *OsMADS8* (nucleotides 616–960 counted from the start codon ATG) and *OsMADS7* (548–911, nucleotide positions from ATG) were used as templates for synthesizing sense and antisense digoxigenin-labeled RNA probes using the DIG Northern Starter Kit (Roche Diagnostics, Mannheim, Germany). The plant material was dehydrated, embedded, sliced (8 μm), pretreated, hybridized, washed and detected as previously described (Kouchi and Hata, 1993).

RNA preparation and gel blot analysis

Total RNA was extracted from rice inflorescences ranging from 3 to 6 cm by using TRIzol reagent (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. The gene-specific probes for *OsMADS7* and *OsMADS8* (Table S2 were labeled with the Prime-a-Gene® Labeling System (Promega Madison, WI, USA). Procedures of gel blot analysis were performed as previously described (Lu *et al.*, 2007).

Small RNA gel blot analysis was performed as described (Liu *et al.*, 2005). A probe corresponding to endogenous MADS-box siRNAs, which contained the *OsMADS8* cDNA coding sequence (nucleotides 48–316 counted from the start codon ATG), was amplified with primers of OsM8M1 and OsM8M2 (Table S2) and cloned into the pGEM-T easy vector (Promega Madison, WI, USA), then transcribed with T7 polymerase *in vitro*, and labeled with [α-^{32}P]ATP.

RT-PCR and real-time PCR expression analysis

Total RNA was isolated as mentioned above. Reverse transcription was performed by using Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo$(dT)_{18}$ primer. The diluted cDNA samples were used as templates for RT-PCR and real-time PCR.

For RT-PCR analysis, the PCR conditions were 94°C for 2 min followed by 22–25 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min; then 10 min of extension at 72°C. For real-time PCR analysis, triplicate quantitative assays were performed using the QuantiTect® SYBR® Green PCR kit with a Rotor-Gene 3000 (Corbett Research, QIAGEN, Hilden, Germany) detection system and software according to the manufacturer's instructions. The amplifying program with the gene-specific primers (Table S2) was as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. The relative expression levels of genes were analysed using the two Standard Curves Relative Quantification method. The amplification of *ACTIN* was used as an endogenous control to normalize all data. All results represent the means of more than three independent experiments.

Yeast two-hybrid (Y2H) analyses

The full-length cDNAs of *OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8* and *OsMADS13* were amplified with gene-specific primers (Table S2). Then the PCR sequences were fused into the activation-domain (AD) vector pGADT7 and the DNA-binding-domain (BD) vector pGBKT7 at the *Eco*RI and *Xho*I/*Sal*I sites, respectively. All constructs were verified by restriction enzyme analyses and sequencing. These constructs were transformed into *Saccharomyces cerevisiae* strain AH109 (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. No detectable self-activation for each of the single construct was observed on SD selective medium (SD-His-Leu+5 mM 3-AT or SD-His-Trp+5 mM 3-AT). The transformants co-transformed with plasmids encoding OsMADS7 (or OsMADS8) and OsMADS13 were used as a positive control (Favaro *et al.*, 2002), and the transformants containing plasmids pGADT7 and pGBKT7 were used as a negative control. Interaction analyses were performed as previously described (Shan *et al.*, 2006).

Coimmunoprecipitation (Co-IP) analyses of the rice SEP-like proteins in Arabidopsis mesophyll protoplasts

Eight vectors, *2*×35SP::6×myc-OsMADS1/*5*/*7*/*8* and *35SP::3*× flag-OsMADS1/*5*/*7*/*8* were constructed to transiently express OsMADS1/5/7/8 in *Arabidopsis* protoplasts for coimmunoprecipitation. Full-length cDNAs of *OsMADS1/5/7/8* were cloned as mentioned above (Y2H), and the fragments were cloned into the vector pRT107–6×myc under the control of double Cauliflower

Mosaic Virus (CaMV) 35S promoter (Töpfer *et al*., 1993) and also into the plasmid pRT105–3×flag driven by a 35S promoter (Zhao *et al*., 2007), respectively. All final constructs were verified by sequencing.

Protoplasts of *A. thaliana* ecotype Columbia (Col-0) were isolated and transformed as described (Yoo *et al*., 2007). Coimmunoprecipitation was done using methods described elsewhere (Zhao *et al*., 2007).

ACKNOWLEDGEMENTS

We thank Dr Bin Liu and Xiaofeng Cao (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for help with the small RNA hybridizations, and Dr Yan Guo (National Institute of Biological Sciences, Beijing) for his kindly gifts of plasmids and excellent technical assistance on Co-IP analysis. Many thank also to two anonymous reviewers whose comments helped considerably to improve our manuscript. This work was supported by the Ministry of Science and Technology of China (Grants 2006CB100202; 2006AA10Z190) and the Chinese Academy of Sciences (Grant KSCX2-YW-R-135).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparison of the wild-type and *OsM7*-RNAi carpels.

Figure S2. Expression analysis of *SEP*-like genes in transgenic lines.

Figure S3. Days to flowering in the *OsM7*/*8*-RNAi and *OsM1*/*5*/*7*/*8*-RNAi transgenic lines.

Figure S4. Expression analyses of two independent lines transformed with the pUJOsM8M construct.

Figure S5. Small interfering RNAs blotting.

Table S1. Sequences of more than 21 nucleotides that are identical among the candidate floral homeotic MADS-box genes.

Table S2. Primers used in this study.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Agrawal, G.K., Abe, K., Yamazaki, M., Miyao, A. and Hirochika, H. (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. *Plant Mol. Biol.* **59**, 125–135.

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 *Silky1* gene reveal conservation in floral organ specification between eudicots and monocots. *Mol. Cell*, **5**, 569–579.

Ampomah-Dwamena, C., Morris, B.A., Sutherland, P., Veit, B. and Yao, J.L. (2002) Down-regulation of *TM29*, a tomato *SEPALLATA* homolog, causes parthenocarpic fruit development and floral reversion. *Plant Physiol.* **130**, 605–617.

Angenent, G.C., Franken, J., Busscher, M., Weiss, D. and van Tunen, A.J. (1994) Co-suppression of the petunia homeotic gene *fbp2* affects the identity of the generative meristem. *Plant J.* **5**, 33–44.

Arora, R., Agarwal, P., Ray, S., Singh, A.K., Singh, V.P., Tyagi, A.K. and Kapoor, S. (2007) MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. *BMC Genomics*, **8**, 242.

Baulcombe, D. (2002) RNA silencing. *Curr. Biol.* **12**, R82–R84.

Baum, D.A., Doebley, J., Irish, V.F. and Kramer, E.M. (2002) Response: missing links: the genetic architecture of flower and floral diversification. *Trends Plant Sci.* **7**, 31–34.

Becker, A. and Theissen, G. (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Mol. Phylogenet. Evol.* **29**, 464–489.

Becker, A., Winter, K.U., Meyer, B., Saedler, H. and Theissen, G. (2000) MADS-Box gene diversity in seed plants 300 million years ago. *Mol. Biol. Evol.* **17**, 1425–1434.

Bowman, J.L., Drews, G.N. and Meyerowitz, E.M. (1991) Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell-types late in flower development. *Plant Cell*, **3**, 749–758.

Cacharron, J., Saedler, H. and Theissen, G. (1999) Expression of MADS box genes *ZMM8* and *ZMM14* during inflorescence development of *Zea mays* discriminates between the upper and the lower floret of each spikelet. *Dev. Genes. Evol.* **209**, 411–420.

Carpenter, R. and Coen, E.S. (1990) Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus*. *Genes Dev.* **4**, 1483–1493.

Chen, Z.X., Wu, J.G., Ding, W.N., Chen, H.M., Wu, P. and Shi, C.H. (2006) Morphogenesis and molecular basis on naked seed rice, a novel homeotic mutation of *OsMADS1* regulating transcript level of *AP3* homologue in rice. *Planta*, **223**, 882–890.

Chuang, C.F. and Meyerowitz, E.M. (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **97**, 4985–4990.

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.

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

Colombo, L., Franken, J., Koetje, E., Vanwent, J., Dons, H.J.M., Angenent, G.C. and Van Tunen, A.J. (1995) The *Petunia* MADS box gene *FBP11* determines ovule identity. *Plant Cell*, **7**, 1859–1868.

Cornejo, M.J., Luth, D., Blankenship, K.M., Anderson, O.D. and Blechl, A.E. (1993) Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol. Biol.* **23**, 567–581.

Cronk, Q.C.B. (2001) Plant evolution and development in a post-genomic context. *Nat. Rev. Genet.* **2**, 607–619.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S. and Yanofsky, M.F. (2004) The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. *Curr. Biol.* **14**, 1935–1940.

Egea-Cortines, M., Saedler, H. and Sommer, H. (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in *Antirrhinum majus*. *EMBO J.* **18**, 5370–5379.

Endress, P.K. (2006) Angiosperm floral evolution: morphological developmental framework. *Adv. Bot. Res.* **44**, 1–61.

Favaro, R., Immink, R.G., Ferioli, V., Bernasconi, B., Byzova, M., Angenent, G.C., Kater, M. and Colombo, L. (2002) Ovule-specific MADS-box proteins have conserved protein–protein interactions in monocot and dicot plants. *Mol. Genet. Genomics*, **268**, 152–159.

Favaro, R., Pinyopich, A., Battaglia, R., Kooiker, M., Borghi, L., Ditta, G., Yanofsky, M.F., Kater, M.M. and Colombo, L. (2003) MADS-box protein complexes control carpel and ovule development in *Arabidopsis*. *Plant Cell*, **15**, 2603–2611.

Flanagan, C.A. and Ma, H. (1994) Spatially and temporally regulated expression of the MADS-box gene *AGL2* in wild type and mutant *Arabidopsis* flowers. *Plant Mol. Biol.* **26**, 581–595.

Frohlich, M.W. (2003) An evolutionary scenario for the origin of flowers. *Nat. Rev. Genet.* **4**, 559–566.

Gómez-Mena, C., de Folter, S., Costa, M.M.R., Angenent, G.C. and Sablowski, R. (2005) Transcriptional program controlled by the floral homeotic gene *AGAMOUS* during early organogenesis. *Development*, **132**, 429–438.

Goto, K. and Meyerowitz, E.M. (1994) Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes Dev.* **8**, 1548–1560.

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

Hannon, G.J. (2002) RNA interference. *Nature*, **418**, 244–251.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Honma, T. and Goto, K. (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature*, **409**, 525–529.

Huang, H., Tudor, M., Weiss, C.A., Hu, Y. and Ma, H. (1995) The *Arabidopsis* MADS-box gene *AGL3* is widely expressed and encodes a sequence-specific DNA-binding protein. *Plant Mol. Biol.* **28**, 549–567.

Huang, J.Q., Wei, Z.M., An, H.L., Xu, S.P. and Zhang, B. (2000) High efficiency of genetic transformation of rice using *Agrobacterium*-mediated procedure. *Acta. Bot. Sin.* **42**, 1172–1178.

Immink, R.G., Tonaco, I.A., de Folter, S., Shchennikova, A., van Dijk, A.D., Busscher-Lange, J., Borst, J.W. and Angenent, G.C. (2009) SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. *Genome Biol.* **10**, R24.

Irish, V.F. (2003) The evolution of floral homeotic gene function. *Bioessays*, **25**, 637–646.

Irish, V.F. and Litt, A. (2005) Flower development and evolution: gene duplication, diversification and redeployment. *Curr. Opin. Genet. Dev.* **15**, 454–460.

Itoh, J., Nonomura, K., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H. and Nagato, Y. (2005) Rice plant development: from zygote to spikelet. *Plant Cell Physiol.* **46**, 23–47.

Jack, T., Brockman, L.L. and Meyerowitz, E.M. (1992) The homeotic gene *APETALA3* of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683–697.

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. *et al.* (2000) *leafy hull sterile1* is a homeotic mutation in a rice MADS box gene affecting rice flower development. *Plant Cell*, **12**, 871–884.

Jofuku, K.D., Denboer, B.G.W., Van Montagu, M. and Okamuro, J.K. (1994) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2. Plant Cell*, **6**, 1211–1225.

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.

Kotilainen, M., Elomaa, P., Uimari, A., Albert, V.A., Yu, D. 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.

Kouchi, H. and Hata, S. (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol. Gen. Genet.* **238**, 106–119.

Lenser, T., Theissen, G. and Dittrich, P. (2009) Developmental robustness by obligate interaction of class B floral homeotic genes and proteins. *PLoS Comput. Biol.* **5**, e1000264.

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.

Liu, B., Li, P.C., Li, X., Liu, C.Y., Cao, S.Y., Chu, C.C. and Cao, X.F. (2005) Loss of function of *OsDCL1* affects microRNA accumulation and causes developmental defects in rice. *Plant Physiol.* **139**, 296–305.

Liu, C., Xi, W., Shen, L., Tan, C. and Yu, H. (2009) Regulation of floral patterning by flowering time genes. *Dev. Cell* **16**, 711–722.

Lu, S.H., Du, X.Q., Lu, W.L., Chong, K. and Meng, Z. (2007) Two *AGAMOUS*-like MADS-box genes from *Taihangia rupestris* (Rosaceae) reveal independent trajectories in the evolution of class C and class D floral homeotic functions. *Evol. Dev.* **9**, 92–104.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1991) *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.* **5**, 484–495.

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.

Malcomber, S.T. and Kellogg, E.A. (2005) *SEPALLATA* gene diversification: brave new whorls. *Trends Plant Sci.* **10**, 427–435.

Mandel, M.A. and Yanofsky, M.F. (1998) The *Arabidopsis AGL9* MADS box gene is expressed in young flower primordia. *Sex. Plant Reprod.* **11**, 22–28.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1992) Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1. Nature*, **360**, 273–277.

Melzer, R. and Theissen, G. (2009) Reconstitution of 'floral quartets' in vitro involving class B and class E floral homeotic proteins. *Nucleic Acids Res.* **37**, 2732–2736.

Melzer, R., Verelst, W. and Theissen, G. (2009) The class E floral homeotic protein SEPALLATA3 is sufficient to loop DNA in floral quartet-like complexes *in vitro*. *Nucleic Acids Res.* **37**, 144–157.

Moon, Y.H., Jung, J.Y., Kang, H.G. and An, G. (1999) Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. *Plant Mol. Biol.* **40**, 167–177.

Münster, T., Deleu, W., Wingen, L.U., Ouzunova, M., Cacharron, J., Faigl, W., Werth, S., Kim, J.T.T., Saedler, H. and Theissen, G. (2002) Maize MADS-box genes galore. *Maydica*, **47**, 287–301.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., 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 type II MADS-box genes in angiosperms. *Proc. Natl Acad. Sci. USA*, **101**, 1910–1915.

Ohmori, S., Kimizu, M., Sugita, M., Miyao, A., Hirochika, H., Uchida, E., Nagato, Y and Yoshida, H. (2009) *MOSAIC FLORAL ORGANS1*, an *AGL6*-like mads box gene, regulates floral organ identity and meristem fate in rice. *Plant Cell*, doi/10. 1105/tpc. 109. 068742.

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

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R. and Yanofsky, M.F. (2001) Conversion of leaves into petals in *Arabidopsis*. *Curr. Biol.* **11**, 182–184.

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 Reprod.* **15**, 113–122.

Petersen, K., Didion, T., Andersen, C.H. and Nielsen, K.K. (2004) MADS-box genes from perennial ryegrass differentially expressed during transition from vegetative to reproductive growth. *J. Plant Physiol.* **161**, 439–447.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E. and Yanofsky, M.F. (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature*, **424**, 85–88.

Pnueli, L., Hareven, D., Broday, L., Hurwitz, C. and Lifschitz, E. (1994) The *TM5* MADS box gene mediates organ differentiation in the three inner whorls of tomato flowers. *Plant Cell*, **6**, 175–186.

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.

Prasad, K., Parameswaran, S. and Vijayraghavan, U. (2005) *OsMADS1*, a rice MADS-box factor, controls differentiation of specific cell types in the lemma and palea and is an early-acting regulator of inner floral organs. *Plant J.* **43**, 915–928.

Riechmann, J.L. and Meyerowitz, E.M. (1997) MADS domain proteins in plant development. *Biol. Chem.* **378**, 1079–1101.

Riechmann, J.L., Krizek, B.A. and Meyerowitz, E.M. (1996) Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. *Proc. Natl Acad. Sci. USA*, **93**, 4793–4798.

Savidge, B., Rounsley, S.D. and Yanofsky, M.F. (1995) Temporal relationship between the transcription of two *Arabidopsis* MADS box genes and the floral organ identity genes. *Plant Cell*, **7**, 721–733.

Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H. and Sommer, H. (1990) Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science*, **250**, 931–936.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H. and Sommer, H. (1992) Characterization of the *Antirrhinum* floral homeotic MADS-box gene *deficiens*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11**, 251–263.

Shan, H., Su, K., Lu, W., Kong, H., Chen, Z. and Meng, Z. (2006) Conservation and divergence of candidate class B genes in *Akebia trifoliata* (Lardizabalaceae). *Dev. Genes. Evol.* **216**, 785–795.

Shitsukawa, N., Tahira, C., Kassai, K., Hirabayashi, C., Shimizu, T., Takumi, S., Mochida, K., Kawaura, K., Ogihara, Y. and Murai, K. (2007) Genetic and epigenetic alteration among three homoeologous genes of a class E MADS box gene in hexaploid wheat. *Plant Cell*, **19**, 1723–1737.

Soltis, P.S. and Soltis, D.E. (2004) The origin and diversification of angiosperms. *Am. J. Bot.* **91**, 1614–1626.

Soltis, D.E., Ma, H., Frohlich, M.W., Soltis, P.S., Albert, V.A., Oppenheimer, D.G., Altman, N.S., Depamphilis, C. and Leebens-Mack, J. (2007) The floral genome: an evolutionary history of gene duplication and shifting patterns of gene expression. *Trends Plant Sci.* **12**, 358–367.

Theissen, G. (2001) Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.* **4**, 75–85.

Theissen, G. and Melzer, R. (2007) Molecular mechanisms underlying the origin and diversification of the angiosperm flower. *Ann. Bot.* **100**, 603–619.

Theissen, G. and Saedler, H. (2001) Plant biology. Floral quartets. *Nature*, **409**, 469–471.

Theissen, G., Kim, J.T. and Saedler, H. (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. *J. Mol. Evol.* **43**, 484–516.

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J.T., Munster, T., Winter, K.U. and Saedler, H. (2000) A short history of MADS-box genes in plants. *Plant Mol. Biol.* **42**, 115–149.

Töpfer, R., Maas, C., Höricke-Grandpierre, C., Schell, J. and Steinbiss, H.H. (1993) Expression vectors for high-level gene expression in dicotyledonous and monocotyledonous plants. *Method Enzymol.* **217**, 67–78.

Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lönnig, W.E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1992) *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of *Antirrhinum* floral organogenesis. *EMBO J.* **11**, 4693–4704.

Uimari, A., Kotilainen, M., Elomaa, P., Yu, D., Albert, V.A. and Teeri, T.H. (2004) Integration of reproductive meristem fates by a *SEPALLATA*-like MADS-box gene. *Proc. Natl Acad. Sci. USA*, **101**, 15817–15822.

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

Whipple, C.J., Ciceri, P., Padilla, C.M., Ambrose, B.A., Bandong, S.L. and Schmidt, R.J. (2004) Conservation of B-class floral homeotic gene function between maize and *Arabidopsis*. *Development*, **131**, 6083–6091.

Whipple, C.J., Zanis, M.J., Kellogg, E.A. and Schmidt, R.J. (2007) Conservation of B class gene expression in the second whorl of a basal grass and outgroups links the origin of lodicules and petals. *Proc. Natl Acad. Sci. USA*, **104**, 1081–1086.

Wikstrom, N., Savolainen, V. and Chase, M.W. (2001) Evolution of the angiosperms: calibrating the family tree. *Proc. Biol. Sci.* **268**, 2211–2220.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature*, **346**, 35–39.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572.

Zahn, L.M., Kong, H., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., DePamphilis, C.W. and Ma, H. (2005) The evolution of the *SEPALLATA* subfamily of MADS-box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. *Genetics*, **169**, 2209–2223.

Zhao, J., Zhang, W., Zhao, Y., Gong, X., Guo, L., Zhu, G., Wang, X., Gong, Z., Schumaker, K.S. and Guo, Y. (2007) SAD2, an importin -like protein, is required for UV-B response in *Arabidopsis* by mediating MYB4 nuclear trafficking. *Plant Cell*, **19**, 3805–3818.

