Genetic Interaction of *OsMADS3*, *DROOPING LEAF*, and *OsMADS13* in Specifying Rice Floral Organ Identities and Meristem Determinacy[1][W][OA]

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Grass plants develop unique floral patterns that determine grain production. However, the molecular mechanism underlying the specification of floral organ identities and meristem determinacy, including the interaction among floral homeotic genes, remains largely unknown in grasses. Here, we report the interactions of rice (*Oryza sativa*) floral homeotic genes, *OsMADS3* (a C-class gene), *OsMADS13* (a D-class gene), and *DROOPING LEAF* (*DL*), in specifying floral organ identities and floral meristem determinacy. The interaction among these genes was revealed through the analysis of double mutants. *osmads13-3 osmads3-4* displayed a loss of floral meristem determinacy and generated abundant carpelloid structures containing severe defective ovules in the flower center, which were not detectable in the single mutant. In addition, in situ hybridization and yeast two-hybrid analyses revealed that *OsMADS13* and *OsMADS3* did not regulate each other's transcription or interact at the protein level. This indicates that *OsMADS3* plays a synergistic role with *OsMADS13* in both ovule development and floral meristem termination. Strikingly, *osmads3-4 dl-sup6* displayed a severe loss of floral meristem determinacy and produced supernumerary whorls of lodicule-like organs at the forth whorl, suggesting that *OsMADS3* and *DL* synergistically terminate the floral meristem. Furthermore, the defects of *osmads13-3 dl-sup6* flowers appeared identical to those of *dl-sup6*, and the *OsMADS13* expression was undetectable in *dl-sup6* flowers. These observations suggest that *DL* and *OsMADS13* may function in the same pathway specifying the identity of carpel/ovule and floral meristem. Collectively, we propose a model to illustrate the role of *OsMADS3*, *DL*, and *OsMADS13* in the specification of flower organ identity and meristem determinacy in rice.

Studies in two model eudicot plants, Arabidopsis (*Arabidopsis thaliana*) and *Antirrhinum majus*, have suggested that MADS box genes play critical roles in regulating flower development. The proposed genetic ABC model explains how three classes of genes (A, B, and C) work together in specifying floral organ identities (Coen and Meyerowitz, 1991). In Arabidopsis, A (*APETALA1* [*AP1*] and *AP2*) alone determines sepals, A and B (*AP3* and *PISTILLATA* [*PI*]) together specify petals, B and C (*AGAMOUS* [*AG*]) define stamens, and C alone defines carpels (Coen and Meyerowitz, 1991). Subsequently, two additional classes of genes (D and E) have been included in the modified ABC model. The D-class genes specify ovules (Angenent et al., 1995), while E-class genes (*SEPALLATA1/2/3/4* [*SEP1/2/3/4*]; formerly named *AGL2/4/9/3*) specify the identity of all four whorls of floral organs and floral meristem determinacy (Pelaz et al., 2000, 2001a, 2001b; Ditta et al., 2004; Liu et al., 2009).

As one of the largest families in flowering plants, the grass family (Poaceae) contains many economically important crops, such as rice (*Oryza sativa*), barley (*Hordeum vulgare*), and maize (*Zea mays*; Linder and Rudall, 2005). These crops have unique floral organization and morphology, which are distinct from those of eudicots and even other monocots (Grass Phylogeny Working Group, 2001; Rudall et al., 2005; Whipple et al., 2007). The spikelet is the structural unit of grass flowers, and each spikelet consists of a varied number of bract-like organs, glumes, and florets. A rice spikelet consists of two pairs of sterile glumes (i.e. rudimentary glumes and empty glumes) and one floret that contains one lemma, one palea in whorl 1, two lodicules in whorl 2 interior to the lemma, six stamens in whorl 3, and a carpel in whorl 4 (Yuan et al., 2009; Zhang and Wilson, 2009).

Although grass flowers are essential for producing grains, the underlying molecular basis that specifies grass floral organs still remains less understood (Clifford, 1987; Whipple et al., 2007). While the ABCDE model is

[1] This work was supported by the National Basic Research Program of China (grant no. 2009CB941500), the National Natural Science Foundation of China (grant no. 30725022), and the Shanghai Leading Academic Discipline Project (grant no. B205).

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www.plantphysiol.org/cgi/doi/10.1104/pp.111.172080

thought to be partially applicable in explaining grass floral development, grasses have diversified genetic components in specifying the identity of floral organs and meristem (Thompson and Hake, 2009). For example, loss-of-function mutants of the orthologs of Arabidopsis *AP3* in maize (*Silky1*) and in rice (*SUPERWOMEN1* [*SPW1*] or *OsMADS16*) display homeotic transformations of stamens to carpels and of lodicules to lemma- or palea-like structures, suggesting the conservation of class B genes between grasses and Arabidopsis (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2007). Grasses have duplicated and subfunctionalized C-class genes (Kramer et al., 2004; Zahn et al., 2006). For example, rice has two *AG* homologs, *OsMADS3* and *OsMADS58* (Kramer et al., 2004). *OsMADS3* plays key roles in both stamen identity specification and late anther development, while *OsMADS58* is crucial for specifying floral meristem determinacy and carpel architecture (Yamaguchi et al., 2006; Hu et al., 2011). Similarly, there is a pair of *AG* homologs in maize: *Zea agamous1* (*zag1*) and *Zea mays mads2* (*zmm2*). The *zag1* gene has been shown to determine floral meristem determinacy, while the biological function of *zmm2* remains unclear (Mena et al., 1996).

In rice, *OsMADS13* is a D-class gene that is orthologous to Arabidopsis *SEEDSTICK* (*STK*) and to *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11* in petunia (*Petunia hybrida*). Cosuppression of *FBP7* and *FBP11* causes the conversion of ovules into carpelloid organs (Colombo et al., 1995). The *osmads13* mutants are associated with homeotic transformation of ovules into carpelloid structures and indeterminate flowers (Dreni et al., 2007; Yamaki et al., 2011). This is in contrast to the mutation of the Arabidopsis *STK* gene, which does not display altered ovule identity (Pinyopich et al., 2003). In Arabidopsis, *AG*, *STK*, *SHATTERPROOF1* (*SHP1*), and *SHP2* are grouped in the monophyletic *AG*-like clade and have been shown to be involved in ovule identity specification. *STK* is the only D-lineage gene and is expressed in the ovule. *stk* single mutants develop a slightly abnormal ovule with a defect in funiculus development, while the *stk shp1 shp2* triple mutant demonstrates the conversion of ovules into leaf-like or carpel-like organs (Favaro et al., 2003; Pinyopich et al., 2003). Furthermore, STK, SHP1, SHP2, and AG were shown to form multimeric complexes in yeast in the presence of SEP MADS box factors, and the defect of ovule development in *sep1/SEP1 sep2 sep3* is similar to that in the *shp1 shp2 stk* triple mutant (Favaro et al., 2002), suggesting the role of Arabidopsis *SEP* genes participating in ovule identity specification. In addition, *AG* was shown to be involved in specifying ovule identity by affecting the expression of *SHP1* and *SHP2* (Brambilla et al., 2007).

The rice *DROOPING LEAF* (*DL*) gene, which is orthologous to Arabidopsis *CRABS CLAW* (*CRC*), encodes a YABBY domain protein and plays a crucial role in specifying the carpel identity and floral meristem determinacy (Yamaguchi et al., 2004). Severe *dl* mutants display complete homeotic transformation of carpels into stamens, while mutations of *CRC* cause abnormal carpel development (Alvarez and Smyth, 1999; Yamaguchi et al., 2004). Moreover, *DL/CRC* interacts antagonistically with class B genes (Alvarez and Smyth, 1999; Yamaguchi et al., 2004), suggesting that *DL* and *CRC* play a conserved and diversified role in controlling carpel identity in rice and Arabidopsis, respectively.

Grasses have diversified *SEP*-like genes, with at least five *SEP*-like members (*OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *OsMADS34*) in rice (Malcomber and Kellogg, 2005; Zahn et al., 2005; Arora et al., 2007). *OsMADS1* (also called *LEAFY HULL STERILE1*) has been characterized as a *SEPALLATA* (*SEP*)-like gene in rice, which is required for specifying the lemma/palea identity and the meristem of inner floral organs (Jeon et al., 2000; Prasad et al., 2001, 2005; Malcomber and Kellogg, 2004; Agrawal et al., 2005; Chen et al., 2006b). Knockdown of both *OsMADS7* and *OsMADS8* results in late flowering, homeotic transformations of lodicules, stamens, and carpels into palea/lemma-like organs, and a loss of floral determinacy. Simultaneous reduction of the expression of four rice *SEP*-like genes, *OsMADS1*, *OsMADS5*, *OsMADS7*, and *OsMADS8*, causes homeotic transformation of all floral organs except the lemma into leaf-like organs (Cui et al., 2010). *OsMADS34* (also called *PANICLE PHYTOMER2*) plays a key role in controlling the development of inflorescences and spikelets in rice (Gao et al., 2010; Kobayashi et al., 2010). Moreover, investigation of the double mutant *osmads34 osmads1* indicates that *OsMADS34* and *OsMADS1* redundantly specify the identities of floral organs, including the lemma/palea, lodicules, stamens, and carpel (Gao et al., 2010). All these data suggest the conserved and diversified functions of rice *SEP*-like genes in specifying flower organ identity. More recently *AGAMOUS-LIKE6* (*AGL6*) genes in monocots and dicots have been also shown to play key roles in specifying floral organ and meristem identity (Hsu et al., 2003; Fan et al., 2007; Ohmori et al., 2009; Reinheimer and Kellogg, 2009; Rijpkema et al., 2009; Thompson et al., 2009; Li et al., 2010; Viaene et al., 2010). *AGL6*-like genes are ancient and widely distributed in gymnosperms and angiosperms and form a sister clade to *SEP*-like genes (Purugganan et al., 1995; Theissen et al., 2000; Becker and Theissen, 2003; Zahn et al., 2005). Mutations in *AGL6* homologous genes in grasses result in defective floral organ identity and meristem determinacy (Ohmori et al., 2009; Thompson et al., 2009; Li et al., 2010).

Although several genes are reported to play roles in specifying flower development in rice, their genetic interactions remain largely unknown. In this study, we characterized the genetic interaction of *OsMADS3*, *DL*, and *OsMADS13* in specifying floral organs and floral meristem determinacy and provided new insights into the molecular mechanisms that regulate flower development in rice.

RESULTS

Identification of New Alleles of *OsMADS13*, *OsMADS3*, and *DL*

To identify rice mutants with floral defects, we screened a population of rice mutants for defective flowers in the *japonica* subspecies 9522 background treated by ^{60}Co γ-ray (280 Gy; Chen et al., 2006a). One mutant line displaying complete female sterility was identified. Genetic analysis and map-based cloning indicated that this mutant has a one-base deletion in the fifth exon in *OsMADS13* (Os012g10540; Supplemental Fig. S1A), causing a frameshift at amino acid 132 and the formation of a premature stop codon. *OsMADS13* expression was specifically reduced in pistils of the mutant (Supplemental Fig. S1B). As the first two mutants of *OsMADS13* (*osmads13-1* and *osmads13-2*) have been reported (Dreni et al., 2007; Yamaki et al., 2011) and a genetic analysis indicated that our mutant is allelic to the reported *osmads13-1*, we named this mutant *osmads13-3*. This mutation is not associated with obvious alteration of the outer three whorl organs, although some *osmads13-3* flowers (31%) displayed three or four stigmas (n = 121; Fig. 1, A and Q) instead of two stigmas in wild-type flowers. Like the *osmads13-1* mutant, *osmads13-3* showed complete female sterility with aborted ovule development (Fig. 1, B and Q) and carpelloid structures (Supplemental Fig. S1, F and G). In addition, the ectopic expression of *DL* was observed in the carpelloid structure of *osmads13-3* (Fig. 2, A–F), suggesting that these ectopic structures have the carpel identity.

Subsequently, we identified a new null mutant of *DL*, called *dl-sup6*, which was allelic with the reported *d1-2* mutant (Nagasawa et al., 2003; Yamaguchi et al., 2004). Sequence analysis showed the insertion of one DNA fragment at the second intron of the *DL* gene (Supplemental Fig. S2A), which abolished the expression of *DL* in the mutant (Supplemental Fig. S2B). Because of five previously identified strong *dl* alleles (*dl-sup1* to *dl-sup5*; Nagasawa et al., 2003; Yamaguchi et al., 2004), we named this mutant *dl-sup6*. Like the severe *dl* mutants, *dl-sup6* displayed a phenotype of drooping leaves (Supplemental Fig. S2C) with ectopic stamens at the position of the carpel (Supplemental Figs. S2, E–H, and 4Q). Some flowers displayed a loss of floral meristem determinacy (Supplemental Fig. S2, F and J). In some cases, ectopic lodicule-like structures or fused anthers were observed in *dl-sup6* (Supplemental Fig. S2, E–G). Scanning electronic microscopy (SEM) observation revealed that *dl-sup6* flowers developed normally at stage Sp6 (Supplemental Fig. S2I). The staging of flowers refers to a previous report (Ikeda et al., 2004). At stage Sp7 or Sp8, *dl-sup6* flowers generated ectopic stamen primordia (Supplemental Fig. S2, G and K). In addition, *dl-sup6* lemmas displayed alternating numbers of vascular tissues, with three, four, or five vascular bundles (Supplemental Fig. S2L), while the wild-type lemma had the characteristic five vascular bundles (Yuan et al., 2009), suggesting an important role of *DL* in specifying lemma identify.

In addition, we recently characterized a new weak allele of *OsMADS3* called *osmads3-4*, which is allelic to *osmads3-1* (Hu et al., 2011). In *osmads3-4*, a two-base deletion was observed in the fifth exon of *OsMADS3*, leading to premature translational termination at amino acid 137 within the K domain. *osmads3-4* flowers developed ectopic lodicule-like structures in whorl 2 and lodicule-like or lodicule-anther mosaic organs in whorl 3 (Fig. 1, C, J, and R). Unlike severe allele *osmads3-3* (Yamaguchi et al., 2006), most *osmads3-4* flowers displayed normal pistil development in the forth whorl (Fig. 1D).

OsMADS3 and *OsMADS13* Synergistically Specify Ovule Identity and Floral Meristem Determinacy

To investigate the genetic interaction between *OsMADS13* and *OsMADS3* in determining rice flower development, we constructed the double mutant *osmads13-3 osmads3-4*. *osmads13-3 osmads3-4* flowers displayed similar developmental defects in the second and third whorls to *osmads3-4* (Fig. 1, E and S). Surprisingly, *osmads13-3 osmads3-4* flowers displayed indeterminate floral development with supernumerary whorls of carpelloid structures without detectable ovule morphology in the flower center (Fig. 1, E–H), which was not observed with the corresponding single mutant. SEM observation showed that *osmads13-3 osmads3-4* floral meristem was similar to that of *osmads3-4* at stage Sp6 during the formation of stamen primordia (Fig. 1, J and K). At early stage Sp8, when the wild-type flower displays one carpel primordium in the fourth whorl and the floral meristem terminates (Fig. 1L), *osmads13-3 osmads3-4* generated both primary and secondary carpel primordia and the floral meristem still persisted (Fig. 1, M and N), suggesting that floral stem cells are not terminated in a timely manner in the double mutant (Fig. 1S). In support of this, the expression of *OSH1*, a maker gene of rice floral meristem (Yamaki et al., 2005), was detectable in the indeterminate floral meristem of *osmads13-3 osmads3-4* at stage Sp8, while the floral meristem in the wild-type flowers had been consumed at the same stage (Fig. 1, O and P). These observations suggest that *OsMADS13* and *OsMADS3* play synergistic roles in ovule development and determinacy of the floral meristem.

To further elucidate the mechanism of *OsMADS13* and *OsMADS3* in floral development, a yeast two-hybrid experiment was performed, and we observed no interaction of these two proteins, as judged by the growth condition in selective culture medium (Supplemental Fig. S3). RNA in situ hybridization analysis indicated that the *OsMADS13* expression pattern was not obviously reduced in *osmads3-4* at stage Sp8, when the ovule forms (Fig. 3, A–C), and the *OsMADS3* mRNA signal was not obviously changed in *osmads13-3* (Fig. 3G). Thus, *OsMADS13* and *OsMADS3* do not seem to influence each other at the transcriptional level.

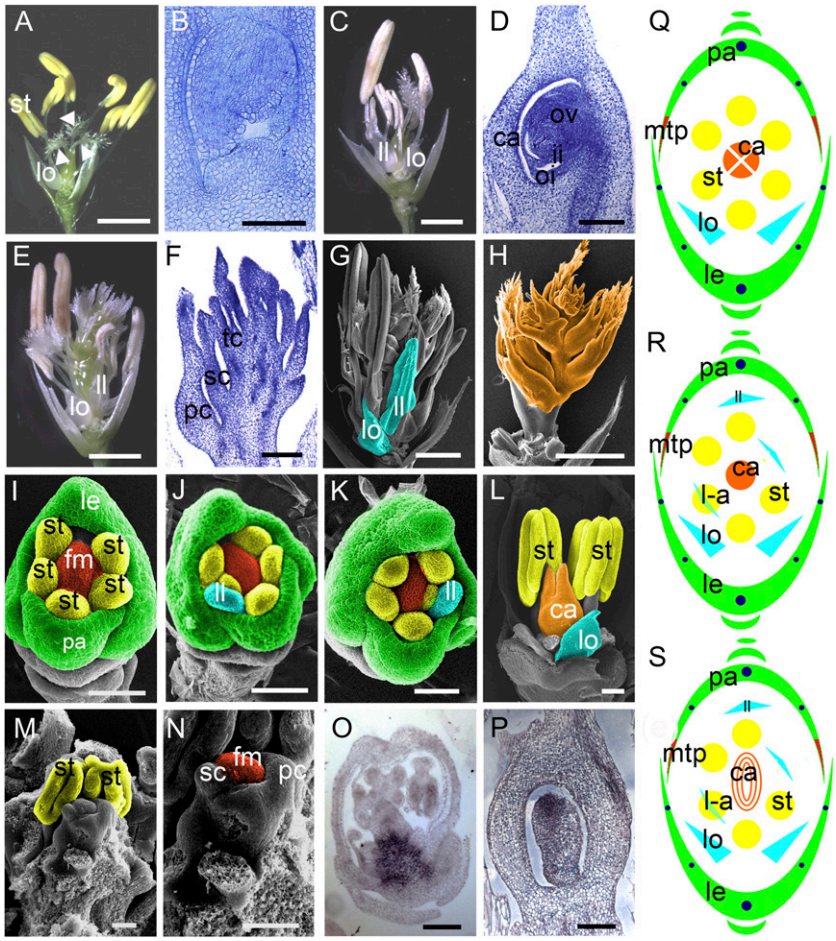

Figure 1. Flower phenotypes of *osmads13-3 osmads3-4*. A, One *osmads13-3* flower with the removal of the lemma and the palea showing normal lodicules and stamens but its pistil with three stigmas. B, Longitudinal section of one *osmads13-3* flower showing abnormal development of the ovule. C, One *osmads3-4* flower with the removal of the lemma and the palea. D, Longitudinal section of one *osmads3-4* flower showing normal development of the carpel and the ovule. E, One *osmads13-3 osmads3-4* flower with the removal of the lemma and the palea showing inner organs. F, Longitudinal section of one *osmads13-3 osmads3-4* flower showing the formation of higher order carpels. G, SEM observation of one *osmads13-3 osmads3-4* flower primordium with the removal of the lemma and the palea. H, SEM observation of one *osmads13-3 osmads3-4* flower primordium displaying the loss of determinacy in the center. I, SEM observation of one *osmads13-3* flower primordium at stage Sp6. J, SEM observation of one *osmads3-4* flower primordium at stage Sp6. K, SEM observation of one *osmads13-3 osmads3-4* flower primordium at stage Sp6. As in J, the primordium of one ectopic organ is emerged. L, SEM observation of a wild-type flower at early stage Sp8 showing the termination of the floral meristem activity. M, SEM observation of one *osmads13-3 osmads3-4* flower at the early stage Sp8 showing the remaining activity of the floral meristem. N, Closeup of M. The primordium of the primary carpel, the secondary carpel, and the floral meristem are indicated. O, Expression pattern of *OSH1* in a wild-type flower at stage Sp8. P, Expression pattern of *OSH1* in one *osmads13-3 osmads3-4* flower at stage Sp8. Q to S, Floral diagrams of *osmads13-3* (Q), *osmads3-4* (R), and *osmads13-3 osmads3-4* (S). ca, Capel; ii, inner integument; l-a, lodicule-anther mosaic organs; ll, lodicule-like structure; lo, lodicules; mtp, marginal tissue of the palea; oi, outer integument; ov, ovule; pc, primary carpel; sc, secondary carpel; st, stamen; tc, tertiary carpel. Bars = 1 mm in A, C, E, and G; 100 μm in B, D, F, and I to L; and 500 μm in H.

OsMADS3 and *DL* Synergistically Terminate the Floral Meristem

To further characterize the potential interaction between *OsMADS3* and *DL* in controlling rice flower development, the double mutant *osmads3-4 dl-sup6* was constructed. Morphological observations indicated that *osmads3-4 dl-sup6* flowers had an altered vascular pattern in the lemma, resembling that of *dl-sup6* (data not shown), suggesting that *DL* controls lemma identity independent of *OsMADS3*. This is consistent with the lack of expression of *OsMADS3* in whorl 1 (Yamaguchi et al., 2006). The floral organs in the second and third whorls of *osmads3-4 dl-sup6* appeared similar to those of *osmads3-4* (Fig. 4, A, B, Q, and R, compared with Fig. 1). Furthermore, *osmads3-4 dl-sup6* developed ectopic floral organ primordia that were similar to those of *osmads3-4* at stage Sp6 (Fig. 4F, compared with Fig. 1G), suggesting that *OsMADS3* functions in lodicule and stamen develop-

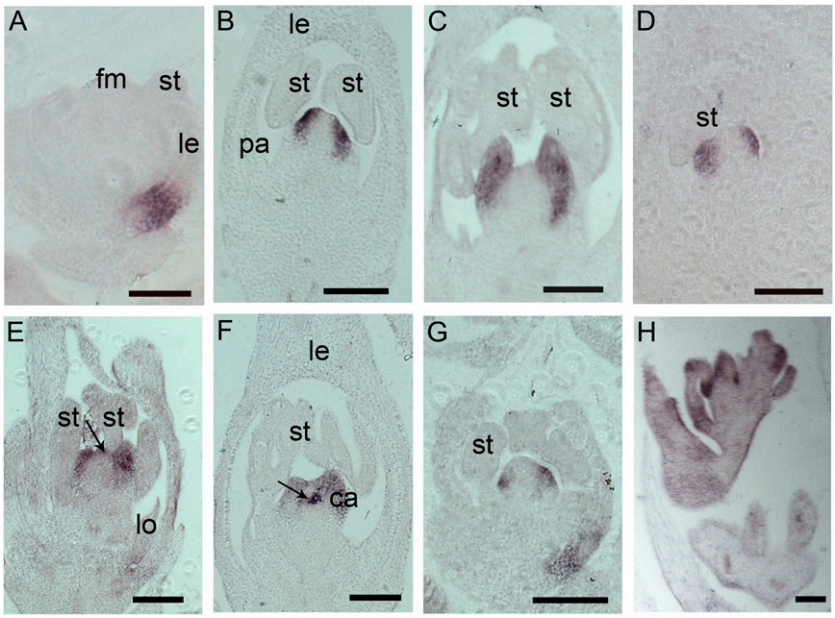

Figure 2. Expression pattern of *DL* in flowers. A to C, In situ hybridization of *DL* mRNA in wild-type flowers. A, At stage Sp6, when the stamen primordia are forming, *DL* transcripts were detected in the midrib of the lemma. B, At stage Sp7, when the carpel primordia are forming, *DL* mRNA was observed in the wild-type carpel primordium. C, At stage Sp8, when the ovule is forming, the expression of *DL* was still observed in the wild-type carpel. D to F, In situ hybridization of *DL* mRNA in *osmads13-3* flowers. D, At stage Sp7, *DL* transcripts were detected in the *osmads13-3* carpel primordium. E, At stage Sp8, *DL* expression signal was observed in the *osmads13-3* carpel (indicated by the arrow). F, At late stage Sp8, *DL* expression was strongly detectable in the indeterminate organ within the carpel in one *osmads13-3* flower. The signal is indicated by the arrow. G, Normal expression of *DL* in *osmads3-4* at stage Sp7. H, Ectopic expression of *DL* in *osmads3-4 osmads13-3* at stage Sp8. ca, Carpel; fm, floral meristem; le, lemma; lo, lodicule; pa, palea; st, stamen. Bars = 50 μm in A and 100 μm in B to H.

ment independent of *DL*. This is in agreement with the fact that *DL* is not expressed in lodicules and stamens (Fig. 2; Yamaguchi et al., 2004). Strikingly, *osmads3-4 dl-sup6* flowers generated supernumerary whorls of undifferentiated lodicule-like organs in the position of the pistil, which seemed to be arranged in bilateral symmetry along the elongated axis (Fig. 4, C–F). In addition, the floral meristem was observed on the top of the axis (Fig. 4E). This phenotype implies a severe loss of floral meristem determinacy, which was further confirmed by the in situ hybridization of *OSH1* mRNA (Fig. 4H). SEM observation showed that at the early stage Sp8, the *osmads3-4 dl-sup6* flower violated the normal development process and formed an indeterminate floral meristem in the flower center (Fig. 4G). Transverse section analysis indicated that these underdeveloped tissues were morphologically close to those of lodicules, with the characteristic pattern of vascular bundles (Fig. 4, I–K). Also, this indication was confirmed by the SEM observation that the morphology of epidermal cells of these underdeveloped tissues appeared similar to that of lodicules (Fig. 4, L and M). Meanwhile, the mRNA of the rice B-class gene *SPW1* (*OsMADS16*), which accumulates in wild-type lodicules and stamens (Fig. 4N; Nagasawa et al., 2003), was detectable in these undifferentiated organs (Fig. 4O). This was combined with the presence of transcripts of the putative class A gene *OsMADS15* (also called *Degenerative Palea* [*DEP*]; Wang et al., 2010a) in the undifferentiated tissues within the flower center of *osmads3-4 dl-sup6* (Fig. 4P). In addition, the normal expression pattern of *DL* was detectable in *osmads3-4* (Fig. 2G) and *OsMADS3* expression was detected in ectopic stamens of *dl-sup6* (Fig. 3H), suggesting that *OsMADS3* and *DL* do not affect the expression of each other at the transcriptional level. These results suggest that *OsMADS3* and *DL* may define the floral meristem in parallel during rice flower development.

Analysis of the Interaction between *OsMADS13* and *DL*

To determine the relationship between *OsMADS13* and *DL*, we constructed the *osmads13-3 dl-sup6* double mutant, and *osmads13-3 dl-sup6* displayed flower defects similar to those of *dl-sup6* (Fig. 5; Supplemental Fig. S2). Moreover, in situ analysis showed that *OsMADS13* transcripts were not obviously detected in *dl-sup6* flowers (Fig. 3D). In contrast, *DL* expression was ectopically observed in the indeterminate organ within the carpel in *osmads13-3* flowers (Fig. 2, D–F). Therefore, we proposed that *OsMADS13* and *DL* may function in the same pathway in specifying carpel/ovule identity and floral determinacy, and *DL* may act upstream of *OsMADS13*, positively regulating *OsMADS13* expres-

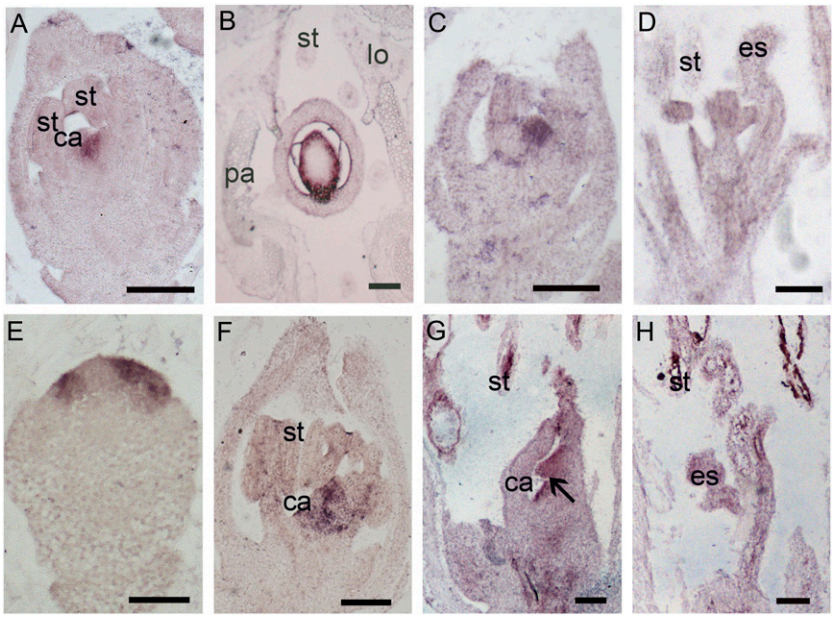

Figure 3. In situ hybridization of *OsMADS13* and *OsMADS3*. A, Longitudinal section of a wild-type flower at early stage Sp8 showing the specific expression of *OsMADS13* in ovule primordium. B, Transverse section of one wild-type flower at late stage Sp8 showing the expression of *OsMADS13* in the ovule. C, The expression of *OsMADS13* in *osmads3-4* at stage Sp8. D, No detectable expression of *OsMADS13* in *dl-sup6*. E, The expression of *OsMADS3* in the wild-type stamen primordia at stage Sp6. F, At stage Sp8, there is detectable expression of *OsMADS3* in the wild-type ovule. G, *OsMADS3* transcripts were observed in the abnormal ovule in *osmads13-3* at stage Sp8. H, *OsMADS3* transcripts in ectopic stamens in *dl-sup6* at stage Sp8. ca, Capel; es, ectopic stamen; fm, floral meristem; lo, lodicules; st, stamen. Bars = 100 μm except for 50 μm in E.

sion, while *OsMADS13* may repress the ectopic expression of *DL* in the ovule.

DISCUSSION

Rice Has a Conserved and Diversified Mechanism Controlling Ovule Identity

Ovule development is of importance in the plant life cycle. The ovule is the source of the megagametophyte and the precursors of seeds, consisting of the nucleus, integument(s), and funiculus (Reiser and Fischer, 1993; Colombo et al., 2008). Previous studies in petunia, Arabidopsis, and rice revealed that the MADS box genes belonging to the *AG* clade are necessary for specifying ovule identity.

In rice, the *AG* clade contains four MADS box members: two C-lineage genes, *OsMADS3* and *OsMADS58*, and two D-lineage genes, *OsMADS13* and *OsMADS21* (Kramer et al., 2004; Zahn et al., 2006). The expression of *OsMADS13* is restricted in the ovule, which is very similar to that of *STK*, *FBP7*, and *FBP11*. In contrast, *OsMADS21* is mainly expressed in developing seeds (Lee et al., 2003; Dreni et al., 2007) and was thought to play a minor role in controlling ovule development (Dreni et al., 2007). Grass species including maize, wheat (*Triticum aestivum*), barley, and rice have duplicated C-class genes (Mena et al., 1996; Kramer et al., 2004; Yamaguchi et al., 2006; Zahn et al., 2006). To date, there is no evidence indicating that class C genes are required for carpel identity in grasses (Thompson and Hake, 2009). In rice, analyses of mutations of *OsMADS3* and knockdown of *OsMADS58* suggested that the two C-class genes have subfunctionalized and redundant functions in rice flower development (Yamaguchi et al., 2006; Hu et al., 2011; M.M. Kater, personal communication; Fig. 6). *osmads3-3* is a strong allele of *OsMADS3*, displaying homeotic transformation of nearly all stamens in whorl 3 into lodicule-like organs, suggesting a major role of *OsMADS3* in stamen specification (Yamaguchi et al., 2006). The intermediate mutant *osmads3-4* displays defective postmeiotic anther development with an abnormal accumulation of reactive oxygen species. OsMADS3 was also shown to directly regulate the expression of *MT-1-4b*, which encodes a type 1 small Cys-rich and metal-binding protein with superoxide anion- and hydroxyl radical-scavenging activity, suggesting that OsMADS3 is a key transcriptional regulator in rice male reproductive development, at least in part by regulating reactive oxygen species homeostasis through *MT-1-4b* (Hu et al., 2011). Previously, *OsMADS58* was shown to play a key role in regulating floral meristem determinacy and normal carpel morphogenesis by the analysis of *OsMADS58* RNA-silenced lines (Yamaguchi et al., 2006). However, a T-DNA insertion knockout mutant of *OsMADS58* was recently identified and showed no obvious floral defects (M.M. Kater, personal communication). The *osmads3-4 osmads58* double mutant displayed more severe defects of inner floral organs and meristem determinacy, suggesting that *OsMADS58* and *OsAMDS3* redundantly regulate inner floral organ identity and flower determinacy (M.M. Kater, personal communication). Therefore, it will be interesting to investigate the genetic interaction of *OsMADS58* with *OsMADS13* and *DL* in the future.

Similarly, two duplicated *AG* homologs (*zag1* and *zmm2*) are present in the maize genome, and mutations in *zag1* cause loss of floral meristem determinacy in the ear, without obvious alteration of floral organ identity (Mena et al., 1996). Currently, no mutants of *zmm2* have

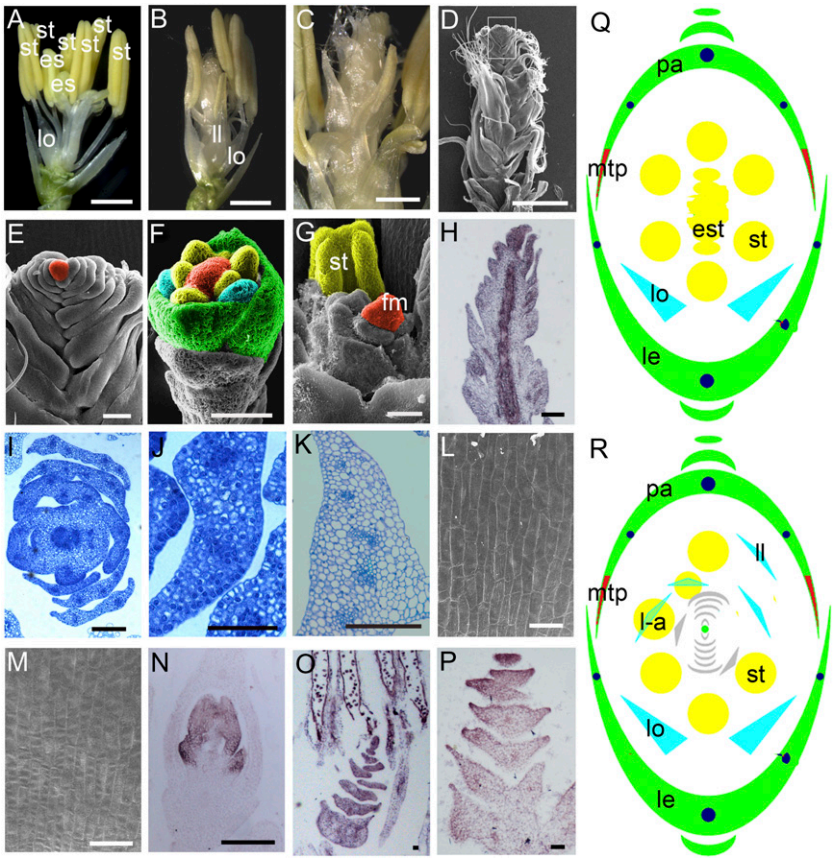

Figure 4. Flower phenotype of *dl-sup6 osmads3-4*. A, One *dl-sup6* flower showing ectopic stamens in the center. B, One *dl-sup6 osmads3-4* flower showing the phenotypes in the second and third whorls similar to *osmads3-4*. C, Closeup of one *dl-sup6 osmads3-4* flower showing mosaic organs and indeterminate organs in the center. D, SEM observation of one *dl-sup6 osmads3-4* flower showing the supernumerary whorls of indeterminate undifferentiated organs in the floral center. E, Closeup of D. F, SEM observation of one *dl-sup6 osmads3-4* flower at stage Sp6. G, SEM observation of one *dl-sup6 osmads3-4* flower after stage Sp7 showing ectopic organs and indeterminate meristem. H, Expression pattern of *OSH1* in the *dl-sup6 osmads3-4* flower at stage Sp8. I and J, Transverse sections of one *dl-sup6 osmads3-4* flower showing the ectopic organs in the flower center. K, Transverse section of one wild-type lodicule. L and M, SEM analysis of epidermal cells of *osmads3-4 du-sup6* ectopic organs and wild-type lodicules, respectively. N, Expression of *SPW1* in wild-type lodicules and stamens. O, Transcripts of *SPW1* detectable in lodicule-like organs of one *dl-sup6 osmads3-4* flower center. P, *OsMADS15* is expressed in lodicule-like organs in one *dl-sup6 osmads3-4* flower center. Q and R, Floral diagrams of *dl-sup6* (Q) and *osmads3-4 dl-sup6* (R). est, Ectopic stamen; fm, floral meristem; l-a, lodicule-anther mosaic organs; ll, lodicule-like structure; lo, lodicules; mtp, marginal tissue of the palea; st, stamen. Bars = 1 mm in A, B, and D; 500 μm in C; 100 μm in E, H to K, and N to P; 50 μm in F; and 20 μm in L and M.

been identified, but the expression pattern of *zmm2* is in agreement with that of class C function (Mena et al., 1996). Here, our genetic analysis of the double mutant *osmads13-3 osmads3-4* indicated that *OsMADS3* plays a critical role in ovule formation and floral meristem determinacy redundantly with *OsMADS13* (Fig. 6). These data also support that the C-class and D-class genes probably retain their functions even though they underwent multiple subfunctionlization events and several neofunctionalizations after duplication within the *AG* clade (Rijpkema et al., 2010).

In rice, the YABBY domain gene *DL* was shown to be crucial for carpel specification (Nagasawa et al., 2003; Yamaguchi et al., 2004), which is different from the well-known ABC genes. In addition, the role of *DL* is distinct from the closely related *YABBY* gene *CRC* of Arabidopsis, which plays a mild role in carpel development (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Yamaguchi et al., 2004). Analysis of *osmads3-4 dl-sup6* flowers indicated that *DL* and *OsMADS3* play a redundant role in terminating floral meristem, but they may function in a distinct pathway (Fig. 6). The ectopic expression of *SPW1* in the supernumerary whorls of lodicule-like organs of the double mutant flower may be explained by the antagonistic role of *DL* in reacting to class B genes in the flower center (Yamaguchi et al., 2004; this study). The ectopic expression of the putative class A gene *OsMADS15* in the floral center may be caused by the mutation of *OsMADS3*. In Arabidopsis and *Antirrhinum*, A- and

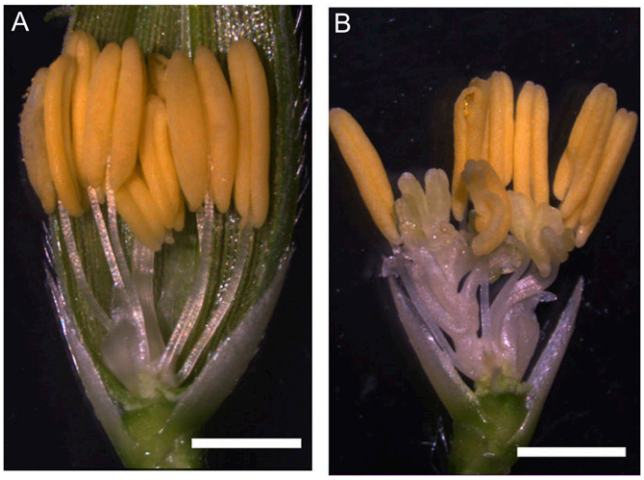

Figure 5. Flower phenotypes of *osmads13-3 dl-sup6*. A, One *osmads13-3 dl-sup6* flower with a weak phenotype, in which several ectopic stamens formed. B, One *osmads13-3 dl-sup6* flower with a severe phenotype.

C-class genes were shown to be antagonistic to each other (Coen and Meyerowitz, 1991). Given the conserved role of the C gene in plant flower development, in combination with the ectopic formation of lodicule-like organs in some *dl-sup6* flowers, we hypothesize that *OsMADS3* and *DL* likely inhibit the expression of putative class A genes such as *OsMADS15* in inner flower organs (Fig. 6).

The rice genome contains four putative A-class genes encoding *AP1/FRUITFULL*-like proteins: *OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS20* (Fornara et al., 2004; Kater et al., 2006; Preston and Kellogg, 2006). However, few class A mutants have been identified in addition to those in Arabidopsis, and the roles of class A genes in floral organ identity are not as clear as was hypothesized by the ABCDE model (Preston and Kellogg, 2006). Unfortunately, besides the *dep* mutant, no other single or double knockout mutant lines for these rice genes have been reported. The *dep* mutant containing a single nucleotide G-to-C substitution at position 94 of the first exon of *OsMADS15* displayed shrunken paleas and slightly elongated lemmas and glumes (Wang et al., 2010a), which are different from the mutant phenotype of class A genes *AP1* and *AP2* in Arabidopsis, with the conversion of sepals into leaf- or bract-like structures and petals into stamen-like organs or loss of sepals (Mandel et al., 1992; Jofuku et al., 1994). Therefore, whether *DEP* functions as an Arabidopsis A-class gene in rice flower development remains to be investigated. AP2 transcription factors in maize and rice have been shown to regulate shoot apical meistem determinacy. In maize, *indeterminate spikelet1* (*ids1*) and the paralog of *ids1*, *sid1*, are required for floral meristem determinacy, and *ids1 sid1* double mutants have no floral meristem, which was replaced by the formation of many bract-like organs, terminating in an ovule-like structure (Chuck et al., 2008). Similarly, mutations in the *ids1*-like gene *SUPERNUMERARY BRACT* in rice result in a delayed transition of spikelet meristem to floral meristem, with additional bract-like organs (Lee et al., 2007).

Furthermore, in this study, our finding suggests that *OsMADS13* and *DL* specify carpel/ovule and floral meristem identity in the same pathway. Besides the observation that *osmads13-3 dl-sup6* displayed flower defects similar to that of *dl-sup6*, no obvious *OsMADS13* expression was detectable in *dl-sup6* flowers, and *DL* transcripts were ectopically detected in *osmads13-3* flowers, suggesting that *DL* may directly or indirectly regulate *OsMADS13* expression. In other words, loss of *OsMADS13* expression in *dl-sup6* may result from the altered carpel/ovule identity in *dl-sup6*, or *DL* regulates carpel/ovule and meristem identity by controlling *OsMADS13* expression. Furthermore, the ectopic ex-

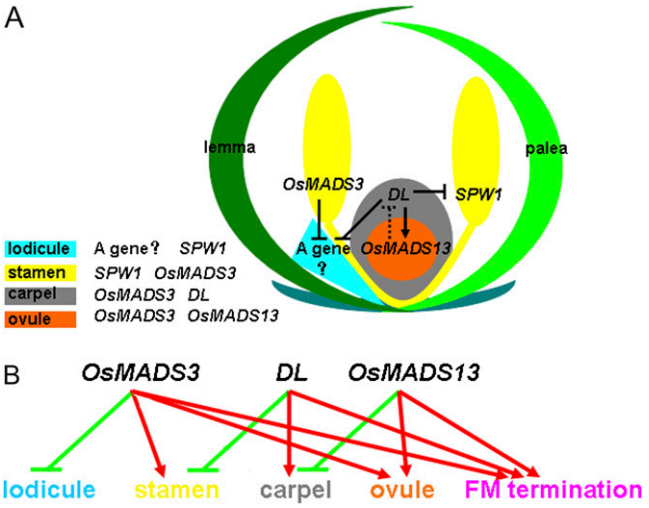

Figure 6. Proposed model to illustrate the genetic interaction between *OsMADS3*, *OsMADS13*, and *DL* in rice flower development. A, Interactions between rice floral organ homeotic genes of A-function genes (such as *OsMADS15*), *SPW1*, *OsMADS3*, *OsMADS13*, and *DL*. Different colors represent the expression patterns of genes in lodicules, stamens, the carpel, and the ovule. *OsMADS3* possibly represses the expression of A-function genes such as *OsMADS15* in the inner floral organs; *DL* may antagonize the expression of *SPW1* and *OsMADS15*. While *OsMADS13* may indirectly limit the expression of *DL* in the ovule, *DL* may directly or indirectly positively regulate *OsMADS13* expression. The broken arrow indicates the possibly indirect or direct regulation of the OsMADS13 expression by *DL*. B, Functions of *OsMADS3*, *DL*, and *OsMADS13* in specifying floral organ identities and floral meristem termination. Green lines and red arrows indicate the functions of repression and promotion, respectively. *OsMADS3* regulates the number of lodicules in whorl 2 by suppressing lodicule development, particularly near the palea (Yamaguchi et al., 2006), represses the formation of lodicules and determines the stamen identity in whorl 3, and specifies ovule identity in the floral center. *DL* represses the formation of stamens and specifies the carpel identity in the flower center, while *OsMADS13* represses carpel formation and determines ovule identity. *OsMADS13* may terminate floral meristem termination in parallel with *OsMADS3*, and *DL* may regulate the floral meristem determinacy in the same pathway of *OsMADS13*. *OsMADS3* and *DL* can redundantly terminate the floral meristem.

pression of *DL* in *osmads13-3* is likely caused by the altered identities of ovule and meristem, and *OsMADS13* may indirectly restrict the expression of *DL* in the ovule (Fig. 6).

Regulation of Rice Floral Meristem Termination

Floral organs are formed by a floral meristem, a pool of pluripotent and dividing cells (Prunet et al., 2009). The regulation of the floral meristem seems to be widely conserved among angiosperms (Ferrario et al., 2004; Prunet et al., 2009). In Arabidopsis, *AG* is a master regulator terminating the floral meristem by turning *WUSCHEL* (*WUS*) off (Sieburth et al., 1998; Sun et al., 2009). In addition to homeotic transformations of stamens into petals, strong *ag* alleles (*ag-1*–*ag-3*) showed a complete loss of floral meristem determinacy, and the carpel was replaced by a new flower (Bowman et al., 1989, 1991; Yanofsky et al., 1990). The genomes of both eudicot and monocot species, including *Antirrhinum*, rice, maize, and barley, contain duplicated and subfunctionalized *AG* homologs (Zahn et al., 2006). Recent analysis of the *osmads3-4 osmads58* double mutant suggests that two rice C-class genes, *OsMADS3* and *OsMADS58*, redundantly regulate floral meristem determinacy (M.M. Kater, personal communication). In *Antirrhinum*, the class C MADS box gene *PLENA* (*PLE*) specifies reproductive organ identity and floral meristem termination, and the phenotype of *ple* mutants is similar to *ag* mutants, with homeotic conversion of reproductive organs to perianth organs (with the exception of nested flowers appearing inside whorl 4 instead of whorl 3 in strong *ag* mutants) and a loss of floral determinacy. In contrast, the mutation of *FARINELLI* (*FAR*), the close paralog of *PLE*, displayed normal flower development only with partial male sterility (Bradley et al., 1993; Davies et al., 1999). Moreover, the B-class MADS box genes *DEF* and *GLO*, which are not normally expressed in the fourth whorl, appeared to be ectopically expressed in *ple far* double mutants, suggesting a distinct role of the C class in *Antirrhinum* genes from that in Arabidopsis in redundantly and negatively regulating the B-function MADS box genes.

It is known that *AG* regulates the floral meristem by indirectly repressing the expression of *WUS* (Lenhard et al., 2001). Recently, *KNUCKLES* (*KNU*) encoding a C2H2 zinc-finger protein was shown to serve as the mediator in this feedback loop (Sun et al., 2009). *AG* directly regulates the expression of *KNU*, which can negatively regulate *WUS* expression (Sun et al., 2009). It remains unclear whether there is a similar mechanism in grasses. In this work, our genetic analyses elucidate the role of *OsMADS3*, *OsMADS13*, and *DL* in floral meristem determinacy (Fig. 6). There are 13 *WOX* (for *WUSCHEL*-related homeobox gene family) members in the rice genome, and *OsWUS* was found to be closely related to the Arabidopsis *WUS* gene (Nardmann and Werr, 2006; Dai et al., 2007; Nardmann et al., 2007; Zhang et al., 2010). But the biological function of *OsWUS* remains unclear. Nardmann and Werr (2006) isolated two *WUS* homologs (*ZmWUS1* and *ZmWUS2*) in maize and rice *OsWUS* and found that they were not expressed in the organizing center of the vegetative shoot apical meristem, as was the *WUS* gene in Arabidopsis.

Similar to the role of eudicot *SEP*-like genes in floral meristem determinacy, grass *SEP*- and *AGL6*-like genes are capable of regulating carpel/ovule development and floral meristem determinacy (Jeon et al., 2000; Prasad et al., 2001, 2005; Agrawal et al., 2005; Chen et al., 2006b; Ohmori et al., 2009; Reinheimer and Kellogg, 2009; Thompson et al., 2009; Cui et al., 2010; Gao et al., 2010; Kobayashi et al., 2010; Li et al., 2010). However, how these genes regulate floral organ identity and meristem determinacy in grasses remains less understood. It is likely that SEP-like and/or AGL6-like proteins act as mediators that constitute multimeric complexes with MADS domain proteins from different clades to regulate flower development in grasses (Immink et al., 2009; Seok et al., 2010; Wang et al., 2010b). In maize, double mutants of the *AGL6*-like gene *bearded-ear* (*bde*) and the class C gene *zag1* display a severe ear phenotype with the conversion of floral meristems to branch-like meristems, which is not detectable in either single mutant, suggesting that *bde* and *zag1* redundantly specify floral meristem identity (Thompson et al., 2009). Moreover, BDE and ZAG1 can physically interact, suggesting that these two proteins act in complexes to control floral development in the maize ear (Thompson et al., 2009). OsMADS7 (also called OsMADS45) and OsMADS8 (also called OsMADS24) were shown to have a similar interaction profile to those of Arabidopsis SEP proteins (Kater et al., 2006; Cui et al., 2010). They can interact with the AG-like protein OsMADS13, which is similar to STK. OsMADS7 and OsMADS8 also interact with Arabidopsis STK and petunia FBP7 (Favaro et al., 2002, 2003).

In summary, this study reveals the genetic interaction of the floral homeotic genes *OsMADS3*, *OsMADS13*, and *DL* and describes an unknown model to illustrate the role of *OsMADS3*, *DL*, and *OsMADS13* in the specification of flower organ identity and meristem determinacy in rice.

MATERIALS AND METHODS

Plant Materials

The mutants *osmads13-3* and *dl-sup6* were identified from an M2 population of rice (*Oryza sativa* subspecies *japonica* '9522') mutagenized with radiation of ^{60}Co γ-ray (Chen et al., 2006a). The strong allele of *OsMADS13* (*osmads13-1*) and the weak allele (*dl-2*) were kindly provided by Prof. Martin M. Kater (Universita degli Studi di Milano) and Prof. Hiro-Yuki Hirano (University of Tokyo), respectively. Prior to the analysis, *osmads13-3*, *osmads3-4*, and *dl-sup6* were all crossed with wild-type 9522 three times. Double mutant plants were isolated by phenotype observation and verified by genotyping with primers 3TPF/3TPR and 13TPF/13TPR for *osmads3-4* and *osmads13-3*, respectively (Supplemental Table S1). Mutant and wild-type rice plants were planted in paddy fields under normal conditions in Shanghai or in a greenhouse at Shanghai Jiao Tong University.

Histological Analysis and Microscopy Observation

Materials were fixed and dehydrated as described by Li et al. (2006). For histological analysis, tissues were substituted by xylene and embedded in Paraplast plus. Then, materials were sectioned to 8 μm thick, stained with toluidine blue, and photographed using a Nikon E600 microscope and a Nikon DXM1200 digital camera. SEM observation was performed with JSM-6360LV (JEOL) as described previously (Li et al., 2006). The dividing of the ovule stages refers to a previous report (Lopez-Dee et al., 1999).

In Situ Hybridization

Treatment of samples was as described previously (Li. et al., 2006). For the construction of specific probes for *OsMADS13*, *SPW1/OsMADS16*, and *DL*, gene-specific fragments of *OsMADS13* cDNA (367–958 bp), *OsMADS16* cDNA (211–686 bp), and *DL* cDNA (121–639 bp) were amplified by reverse transcription (RT)-PCR using primers 13PPF/13PPR, 16PPF/16PPR, and DLPPF/DLPPR, respectively (Supplemental Table S1) and cloned into pBluescript II KS+ phagemid vector (Stratagene). The probe construct of *OSH1* was generated as described previously (Agrawal et al., 2005; Yamaki et al., 2005; Li et al., 2010). Construction of the *OsMADS3* and *OsMADS15* probes referred to previous reports (Kyozuka et al., 2000; Yamaguchi et al., 2006). Digoxigenin-labeled antisense and sense probes were transcribed in vitro as described previously (Chu et al., 2006). Images were obtained using the Olympus Nikon E600 microscope.

Yeast Two-Hybrid Analysis

The MATCHMAKER GAL4 Two-Hybrid System (Clontech) was used to detect the interaction between OsMADS3 and OsMADS13. cDNA fragments encoding the IKC domain of OsMADS3 and OsMADS13 were amplified by RT-PCR with primers 3YF/3YR and 13YF/13YR, respectively (Supplemental Table S1), and the cDNA fragment encoding the IKC14 domain of OsMADS6 was amplified by RT-PCR with primers 6YF/6YR (Supplemental Table S1). Then, these cDNA fragments were cloned into pGBKT7 and pGADT7 to fuse with the BD (bait domain) and AD (activation domain) of GAL4, respectively. Recombinant vectors were named AD-13, BD-13, AD-3, BD-3, AD-6, and BD-6 respectively. Self-activation was assayed on selective synthetic dropout medium plates (−Leu/−His/+3-amino-1,2,4-triazole [3-AT] or −Trp/−His/+3-AT). Then, combinations of AD-3/BD-13 and BD-3/AD-13 were transformed into yeast strain AH109 simultaneously according to the protocol. The transformants cotransformed with plasmids encoding OsMADS6 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. The interaction was judged by the growth condition on selective mediums (−Trp/−Leu/−His/+3-AT) according to the protocol from the company.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Schematic representation of *osmads13* mutants and abnormal ovule development of *osmads13-3*.

Supplemental Figure S2. Schematic representation of *dl-sup* mutants and the phenotype of *dl-sup6*.

Supplemental Figure S3. OsMADS3 does not interact with OsMADS13 in yeast cells.

Supplemental Table S1. Primers used in this research.

ACKNOWLEDGMENTS

We acknowledge Prof. Martin M. Kater for providing the allele of *osmads13-1* and Prof. Hiro-Yuki Hirano for the weak allele of *dl-2*. We thank the anonymous reviewers for comments on the manuscript, Dr. Hao Yu and Dr. Ning Jiang for editing the manuscript, and Z.J. Luo and M.J. Chen for genetic mutant screening.

Received January 6, 2011; accepted March 24, 2011; published March 28, 2011.

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