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[#]Corresponding author: Dabing Zhang

Address: School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China;

Tel: 0086-21-34205073; Fax: 0086-21-34204869;

E-mail: zhangdb@sjtu.edu.cn.

Genetic interaction of *OsMADS3, DROOPING LEAF* and *OsMADS13* in specifying rice floral organs identities and meristem determinacy

Haifeng Li[1*], Wanqi Liang[1*], Changsong Yin[1], Lu Zhu[1], Dabing Zhang[1, 2#]

[1]School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

[2]Bio-X Center, Key Laboratory of Genetics & Development and Neuropsychiatric Diseases, Ministry of Education, Shanghai Jiao Tong University, Shanghai 200240, China

[#]Corresponding author: Dabing Zhang

Address: School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China.

Tel: 0086-21-34205073; Fax: 0086-21-34204869.

E-mail: zhangdb@sjtu.edu.cn.

*These authors contributed equally to this study.

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*These authors contributed equally to this study.

#Corresponding author: Dabing Zhang, e-mail: zhangdb@sjtu.edu.cn.

Abstract

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.

Key words: rice; flower organ identity; meristem termination; genetic interaction; homeotic genes

Introduction

Studies in two model eudicot plants *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*; *AP2*) alone determines sepals, A and B (*AP3*; *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; Pelaz et al., 2001a; Pelaz et al., 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). 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 thought to be partially applicable in explaining the 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 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: *zag1* (*zea agamous1*) and *zmm2* (*Zea mays mads2*). 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 which is orthologous to *Arabidopsis SEEDSTICK* (*STK*), and *FLORAL BINDING PROTEIN 7* (*FBP7*) and *FBP11* in petunia. Co-suppression 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., 2010). 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*, *SHP1* (*SHATTERPROOF1*) and *SHP2* are grouped in the monophyletic *AG*-like clade and have been shown to be involved in the ovule identity specification. *STK* is the only D-lineage gene and expressed in the ovule. *stk* single mutants develop a slightly abnormal ovule with a defect of the funiculus development, while *stk shp1 shp2* triple mutant demonstrate 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 *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*) gene, encodes a YABBY domain protein, 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*, *LHS1*) 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; Malcomber and Kellogg 2004; Agrawal et al., 2005; Prasad et al., 2005; Chen et al., 2006a). Knock down 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*, *PAP2*) 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 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 (*Oryza sativa* L. ssp. *Japonica*) treated by ^{60}Co γ-ray (280 Gy) (Chen et al., 2006b). One mutant line displaying complete female sterility was identified. Genetic analysis and map-based cloning indicated that this mutant has one base deletion in the fifth exon in *OsMADS13* (Os012g10540) (Figure S1A), causing a frameshift at 132th amino acid and the formation of premature stop-codon. *OsMADS13* expression was specifically reduced in pistils of the mutant (Figure S1B). As the first two mutants of *OsMADS13* (*osmads13-1* and *osmads13-2*) have been reported (Dreni et al., 2007; Yamaki et al., 2010), and a genetic analysis indicated that our mutant is allelic to the reported *osmads13-1*, we named this mutant as *osmads13-3*. This mutation is not associated

with obvious alteration of outer three whorl organs, some *osmads13-3* flowers (31%) displayed three or four stigmas (n=121) (Figures 1A and 1Q), instead of two stigmas in wild-type flowers. Like the *osmads13-1* mutant, *osmads13-3* showed complete female sterility with aborted ovule development (Figures 1B and 1Q) and carpelloid structures (Figures S1F and S1G). In addition, the ectopic expression of *DL* was observed in the carpelloid structure of *osmads13-3* (Figures 2A to 2F), 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 (Figure S2A) which abolished the expression of *DL* in the mutant (Figure 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 (Figure S2C), with ectopic stamens at the position of the carpel (Figures S2E-S2H and 4Q). Some flowers displayed a loss of floral meristem determinacy (Figures S2F and S2J). In some cases, ectopic lodicule-like structures or fused anthers were observed in *dl-sup6* (Figures S2E, S2F and S2G). Scanning electronic microscope (SEM) observation revealed that *dl-sup6* flowers developed normally at stage Sp6 (Figure S2I). The staging of flower stage refers to previous report (Ikeda et al., 2004). At stage Sp7 or Sp8, *dl-sup6* flowers generated ectopic stamen primordia (Figures S2G and S2K). In addition, *dl-sup6* lemmas displayed alternated numbers of vascular tissues, three, four or five vascular bundles (Figure S2L), while the wild-type lemma has 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 2-base deletion was observed in the fifth exon of *OsMADS3*, leading to premature translational termination at the 137th amino acid 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 (Figures 1C, 1J and 1R). Unlike severe allele *osmads3-3* (Yamaguchi et al., 2006), most of *osmads3-4* flowers displayed normal pistil development in the forth whorl (Figure 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 double mutant *osmads13-3 osmads3-4*. *osmads13-3 osmads3-4* flowers displayed similar developmental defects in the second and third whorls to *osmads3-4* (Figures 1E and 1S). Surprisingly, *osmads13-3 osmads3-4* flowers displayed indeterminate floral development with supernumerary whorls of carpelloid structures without detectable ovule morphology in the flower center (Figures 1E to 1H), 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 (Figures 1J and 1K). At early stage Sp8 when the wild-type flower displays one carpel primordium in the forth whorl and floral meristem terminates (Figure 1L), *osmads13-3 osmads3-4* generated both primary and secondary carpel primordia, and floral meristem still persisted (Figures 1M and 1N), suggesting that floral stem cells are not timely terminated in the double mutant (Figure 1L). Supportively, 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 has been consumed at the same stage (Figures 1O and 1P). 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, yeast two hybridization experiment was performed, and we observed no interaction of these two proteins judged by the growth condition in selective culture medium (Figure S3). RNA *in situ* hybridization analysis indicated that the *OsMADS13*

expression pattern was not obviously altered in *osmads3-4* at stage Sp8 when the formation of ovule (Figures 3A-3C), and *OsMADS3* mRNA signal was not obviously changed in *osmads13-3* (Figure 3G). Thus *OsMADS13* and *OsMADS3* do not seem to influence each other at transcriptional level.

OsMADS3 and *DL* synergistically terminate floral meristem

To further characterize the potential interaction between *OsMADS3* and *DL* in controlling rice flower development, double mutant *osmads3-4 dl-sup6* was constructed. Morphological observations indicated that *osmads3-4 dl-sup6* flowers had altered vascular pattern in the lemma, resembling that of *dl-sup6* (date not shown), suggesting that *DL* controls lemma identity independent of *OsMADS3*. This is consistent with the lack of expression of *OsMADS3* in the whorl 1 (Yamaguchi et al., 2006).The floral organs in the second and third whorls of *osmads3-4 dl-sup6* appeared similar to that of *osmads3-4* (Figures 4A, 4B, 4Q and 4R, compared with Figure 1). Furthermore, *osmads3-4 dl-sup6* developed ectopic floral organ primordia which were similar to that of *osmads3-4* at stage Sp6 (Figure 4F, compared with Figure 1G), suggesting that *OsMADS3* functions in lodicule and stamen development independent of *DL*. This is in agreement with the fact that *DL* is not expressed in lodicules and stamens (Figure 2; Yamaguchi et al., 2004). Strikingly, *osmads3-4 dl-sup6* flowers generated supernumerary whorls of undifferentiated lodicule-like organs in the position of pistil, which seemed to be arranged in bilateral symmetry along the elongated axis (Figures 4C to 4F). In addition, the floral meristem was observed on the top of axis (Figure 4E). This phenotype implies a severe loss of floral meristem determinacy which was further confirmed by the *in situ* hybridization of *OSH1* mRNA (Figure 4H). SEM observation showed that at early stage of Sp8, the *osmads3-4 dl-sup6* flower violated from the normal development process and formed indeterminate floral meristem in the flower center (Figure 4G).Transverse section analysis indicated that these underdeveloped tissues were morphologically close to those of lodicules with the characteristic pattern of vascular bundles (Figures 4I to 4K). Also this indication was confirmed by the SEM observation that the morphology

of epidermal cells of these underdeveloped tissues appeared similar to those of lodicules (Figures 4L and 4M). Meanwhile, the mRNA of rice B-class gene *SPW1* (*OsMADS16*), which accumulates in wild-type lodicules and stamens (Figure 4N; Nagasawa et al., 2003), was detectable in these undifferentiated organs (Figure 4O). This was combined with the presence of transcripts of the putative class A gene *OsMADS15* (also called *Degenerative Palea*, *DEP*) (Wang et al., 2010) in the undifferentiated tissues within the flower center of *osmads3-4 dl-sup6* (Figure 4P). In addition, normal expression pattern of *DL* was detectable in *osmads3-4* (Figure 2G), and *OsMADS3* expression was in normal and ectopic stamens of *dl-sup6* (Figure 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 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 that of *dl-sup6* (Figure 5; Supplemental Figure 2). Moreover, in situ analysis showed that *OsMADS13* transcripts were not obviously detected in *dl-sup6* flowers (Figure 3D). In contrast, *DL* expression was ectopically observed in the indeterminate organ within the carpel in *osmads13-3* flowers (Figures 2D-2F). 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* expression, while *OsMADS13* may repress the ectopic expression of *DL* in the ovule.

Discussion

Rice has conserved and diversified mechanism controlling the ovule identity

The ovule development is of importance in plant life cycle. 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*, 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 function in rice flower development (Yamaguchi et al., 2006; Hu et al., 2011; Dreni et al., unpublished data) (Figure 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 (ROS). OsMADS3 was also shown to directly regulate the expression of *MT-1-4b*, which encodes a type 1 small cysteine-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 ROS 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 knock-out mutant of *OsMADS58* was recently identified and showed no obvious floral defects (Kater et al.,

personal communication). *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 organs identity and flower determinacy (Kater et al., personal communication). Therefore it will be interesting to investigate the genetic interaction of *OsMADS58* with *OsMADS13* and *DL*, respectively in the future.

Similarly, two duplicated *AG* homologs (*zag1* and *zmm*2) 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 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 double mutant *osmads13-3 osmads3-4* indicated that *OsMADS3* plays a critical role in ovule formation and floral meristem determinacy redundantly with *OsMADS13* (Figure 6). These data also support that the C-class and D-class genes probably retain their function even though they underwent multiple subfunctionlization events and several neofunctionalization after duplication within *AG* clade (Rijpkema et al., 2010).

In rice a YABBY domain gene *DL* was shown to be crucial for carpel specification (Nagasawa et al., 2003; Yamaguchi et al., 2004), that 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 (Yamaguchi et al., 2004; Alvarez and Smyth, 1999; Bowman and Smyth, 1999). 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 distinct pathway (Figure 6). The ectopic expression of *SPW1* in the supernumerary whorls of lodicules-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 maybe caused by the mutation of *OsMADS3*. In *Arabidopsis* and *Antirrhinum*, A and C class genes were shown to be antagonistic to each other (Coen and Meyerowitz, 1991). Given that the conserved role of C gene in plant flower development, in combination with the

ectopic formation of lodicules-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 (Figure 6).

Rice genome contains four putative A class genes encoding *AP1/FRUITFULL* (*FUL*)-like proteins, *OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS20* (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 that was hypothesized by the ABDCE model (Preston and Kellogg, 2006). Unfortunately, beside the *dep* mutant, no other single or double knock-out 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., 2010), 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 *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, *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* (*SNB*) in rice result in delayed transition of shoot SM 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. Beside 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 be resulted from the altered carpel/ovule identity in *dl-sup6*, or *DL* regulates carpel/ovule and meristem identity by controlling the *OsMADS13* expression. Furthermore, the ectopic expression 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 (Figure 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 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 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* to *-3*) showed a -complete loss of floral meristem determinacy, and the carpel is replaced by a new flower (Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991). 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 *osmads3-4 osmads58* double mutant suggests that two rice C class genes *OsMADS3* and *OsMADS58* redundantly regulate the floral meristem determinacy (Kater et al., 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 appeared inside the whorl 4 instead of the 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 partially 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 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 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* that can negatively regulate the *WUS* expression (Sun et al., 2009). It remains unclear whether there is a similar mechanism exists in grasses. In this work, our genetic analyses elucidate the role of *OsMADS3*, *OsMADS13* and *DL* in floral meristem determinacy (Figure 6). There are 13 *WOX* (*WUSCHEL*-related homeobox gene family) members in rice genome, *OsWUS* was found to be closely related to *Arabidosis 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 (SAM) as 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; Agrawal et al., 2005; Prasad et al., 2005; Chen et al., 2006a; Ohmori et al., 2009; Thompson et al., 2009; Reinheimer and Kellogg 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; Wang et al., 2010; Seok et al., 2010). In maize, double mutants of *AGL6*-like gene *bearded-ear* (*bde*) and 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 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 AG-like protein OsMADS13 which is similar to STK. OsMADS7 and OsMADS8 also interact with *Arabidopsis* STK and Petunia FBP7 (Favaro et al., 2002; Favaro et al., 2003).

In summary, this study reveals the genetic interaction of 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

Plants materials

The mutants *osmads13-3* and *dl-sup6* were identified from M2 population of 9522 (*Oryza sativa* L. ssp. *Japonica* cv.9522) mutagenized with radiation of γCo^{60} (Chen et al., 2006b). The strong allele of *OsMADS13* (*osmads13-1*) and weak allele (*dl-2*) were kindly provided by Professor Martin M. Kater (Universita` degli Studi di Milano, Italy) and Professor Hiro-Yuki Hirano (University of Tokyo, Japan) respectively. Prior to the analysis, *osmads13-3*, *osmads3-4* and *dl-sup6* were all crossed with wild-type 9522 three times respectively. Double mutant plants were isolated by phenotype observation and verified by genotyping with primers3TPF/3TPR and 13TPF/13TPR for *osmads3-4* and *osmads13-3*, respectively (Supplemental Table 1). Mutant and wild-type rice plants were planted in paddy fields under normal condition in shanghai or greenhouse in Shanghai Jiao Tong University, China.

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 and stained with toluidine blue and photographed using a Nikon E600 microscope (Nicon Corporation) and a Nikon DXM1200 digital camera (Nicon Corporation). Scanning electron microscopy (SEM)

observation was performed with JSM-6360LV (JEOL) as described previously (Li et al., 2006). The dividing of the ovule stage refers to previous report (Lopez-Dee et al., 1999).

In situ hybridization

Treatment of samples was as described previously (Li. et al., 2006). For 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 RT-PCR using primers13PPF/13PPR, 16PPF/16PPR and DLPPF/DLPPR respectively (Supplemental Table 1) and cloned into pBluescript II KS+ phagemid vector (Stratagene). Probe construct of *OSH1* was generated as described previously (Agrawal et al., 2005; Yamaki et al., 2005; Li et al., 2010). Construction of *OsMADS3* and *OsMADS15* probe referred to previous report (Yamaguchi et al., 2006; Kyozuka et al., 2000). Digoxygenin-labeled antisense and sense probes were transcribed *in vitro* as described previously (Li et al., 2006). Images were obtained using the Olympus Nikon E600 microscope.

Yeast two hybridization

The MATCHMAKER GAL4 Two-Hybrid System (CLONTECH, Japan) was used to detect the interaction between OsMADS3 and OsMADS13. cDNA fragments encoding IKC domain of OsMADS3 and OsMADS13 were amplified by RT-PCR with primers 3YF/3YR, 13YF/13YR respectively (Supplemental Table 1), and cDNA fragment encoding IKC14 domain of OsMADS6 was amplified by RT-PCR with primers 6YF/6YR (Supplemental Table 1). 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, BD-6 respectively. Self activation was assayed on SD plates (-Leu/-His/+3-AT or -Trp/-His/+3-AT). Then, combination of AD-3, BD-13 and BD-3, AD-13 was transformed into yeast strain AH109 simultaneously according to the protocol. The transformants co-transformed 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.

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Figure Legends

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 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 Sp6. Like in J, the primordium of one ectopic organ is emerged.

L, SEM observation of wild-type flower at early stage Sp8 showing the termination of the floral meristem activity.

M, SEM observation of one *osmads13-3 osmads3-4* at the early stage of Sp8 showing the remaining activity of floral meristem. The primordium of the primary carpel, the secondary carpel and the floral meristem were indicated by blue, red and green arrows respectively.

N, Close-up of M.

O, Expression pattern of *OSH1* in wild type flower at stage Sp8.

P, Expression pattern of *OSH1* in *osmads13-3 osmads3-4* flower at stage Sp8.

Q-S, Florla diagram of *osmads13-3* (Q), *osmads3-4* (R) and *osmads13-3 osmads3-4* (S).

Pc, primary carpel; sc, secondary carpel; tc, tertiary carpel; st, stamen; ca, capel; lo, lodicules; ll, lodicules-like structure; l-a, lodicules-anther mosaic organs; ov, ovule; oi, outer integument; ii, inner integument; mtp, marginal tissue of the palea.

Bars= 1mm in A, C, E and G; 100 μm in B, D, Fand I-L and 500 μm in H.

Figure 2. Expression pattern of *DL* in flowers.

A-C, *In situ* hybridization of *DL* mRNA in wild-type flowers.

A, At stage Sp6 when the formation of stamen primordia, *DL* transcripts were detected in the midrib of the lemma.

B, At stage Sp7 when the formation of carpel primordia, *DL* mRNA was observed in the wild-type carpel primordium.

C, At stage Sp8 when the formation of ovule, the expression of *DL* was still observed in the wild-type carpel.

D-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.

Pa, palea; le, lemma; fm, floral meristem; st, stamen; ca, carpel; lo, lodicule. Bars=50 μm in A, 100 μm in B to H.

Figure 3. *In situ* hybridization of *OsMADS13* and *OsMADS3*.

A, Longitudinal section of 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 of 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, the 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.

st, stamen; ca, capel; lo, lodicules; es, ectopic stamen; fm, floral meristem. Bars=100 μm except 50 μm in E.

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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, Close-up 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, Close-up 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 section 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 lodicules-like organs of *dl-sup6 osmads3-4*

flower center.

P, *OsMADS15* is expressed in lodicules-like organs in *dl-sup6 osmads3-4* flower center.

Q and R, Floral diagrams of *dl-sup6* (Q) and *osmads3-4 dl-sup6* (R).

st, stamen; est, ectopic stamen; lo, lodicules; ll, lodicules-like structure; l-a, lodicules-anther mosaic organs; mtp, the marginal tissue of the palea; fm, floral meristem.

Bars=1 mm in A, B and D; 500 μm in C; 100 μm in E, H-K, N-P; 50 μm in F; 20 μm in L and M.

Figure 5. Flower phenotypes of *osmads13-3 dl-sup6.*

A, One *osmads13-3 dl-sup6* flower with weak phenotype in which several ectopic stamens formed.

B, One *osmads13-3 dl-sup6* flower with severe phenotype.

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 gene(s) (such as *OsMADS15*), *SPW1*, *OsMADS3*, *OsMADS13* and *DL*. Different colors represent expression pattern of genes in lodicules, stamens, the carpel and the ovule, respectively. *OsMADS3* possibly represses the expression of A-function gene(s) such as *OsMADS15* in the inner floral organs; *DL* may antagonize the expression of *SPW1* and *OsMADS15* respectively. While *OsMADS13* may indirectly limit the expression of *DL* in the ovule, and *DL* may directly or indirectly positively regulates the *OsMADS13* expression.

B, Functions of *OsMADS3, DL and OsMADS13* in specifying floral organ identities and floral meristem termination. Green lines and red arrows indicate the function of repression and promotion, respectively. Broken arrow means the possibly indirect or direct regulation of the *OsMADS13* expression by *DL*. *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

determinates the stamen identity in whorl 3, and specifies ovule identity in the floral center, respectively. *DL* represses the formation of stamens and specifies the carpel identity in the flower center, while *OsMADS13* represses the carpel formation and determines the ovule identity. *OsMADS13* may terminate floral meristem termination in parallel with *OsMADS3*, *DL* may regulate the floral meristem determinacy in the same pathway of *OsMADS13*. *OsMADS3* and *DL* can redundantly terminate the floral meristem.

Supplemental Figure 1. Schematic representation of *osmads13* mutants and abnormal ovule development of *osmads13-3*.

A, Schematic representation of *osmads13* mutants.

B, Reduced expression of *OsMADS13* in *osmads13-3*.

C, One wild-type flower at stage Ov2 when the megasporogenesis starts with the formation of the distinctive archespore indicated by the arrow as well as the initiation of outer and inner integument primordia.

D, One wild-type flower at stage Ov4 when the inner integument encloses the nucellus exept for the micropyle.

E, One wild-type flower at stage Ov9 when the embryo sac is mature.

F, One *osmads13-3* flower at stage Ov2 displayed the ectopic carpel within the primary carpel.

G, One *osmads13-3* flower at stage Ov9 with indeterminate floral organ at the position of the wild-type ovule.

Ca, carpel; nu, nucellus; ar, archespore; ii, inner integument; oi, outer integument; mic, micropylar pole; es, embryo sac; cl, carpel like structure; ovule developmental stages refer to Lopez et al (1999).

Bars= 200 μm in C to G.

Supplemental Figure 2. Schematic representation of *dl-sup* mutants and phenotype of *dl-sup6*.

A, Schematic representation of *dl-sup* mutants.

B, No detectable *DL* expression in *dl-sup6* leaves.

C, One *dl-sup6* plant displaying drooping leaves.

D, One *dl-sup6* spikelet at stage Sp8.

E, SEM analysis of one *dl-sup6* flower at stage Sp8 with severe floral defects. The lemma and palea were ripped off to show the inner floral organs.

F, One *dl-sup6* flower at stage Sp8 displaying loss of floral determinacy in the floral center revealed by SEM analysis.

G, One *dl-sup6* flower at stage Sp8 with weak floral defects revealed by SEM analysis. The lemma and palea were ripped off to show the inner floral organs. Arrowheads in F and G indicate the fused anthers.

H, Transverse section of *dl-sup6* flower to show normal stamens in the third whorl and ectopic organs in the center. Normal stamens and ectopic stamens are indicated by red and green asterisks respectively.

I, SEM analysis revealed normal floral development *dl-sup6* at stage Sp6.

J and K, SEM analysis showed primordia of ectopic organs in *dl-sup6* flowers with strong and weak phenotyps at early stage of Sp8, respectively.

L, Transverse sections of *dl-sup6* showing three (left), four (middle) and five (right) vascular bundles, respectively, in the lemma.

Arrows in E and F indicate the lodicules-like organs in flower center. Le, lemma; pa, palea; ll, lodicules-like organs; lo, lodicules. Bars=1mm in D to G; 100 μm in H, J-L; and 50 μm in I.

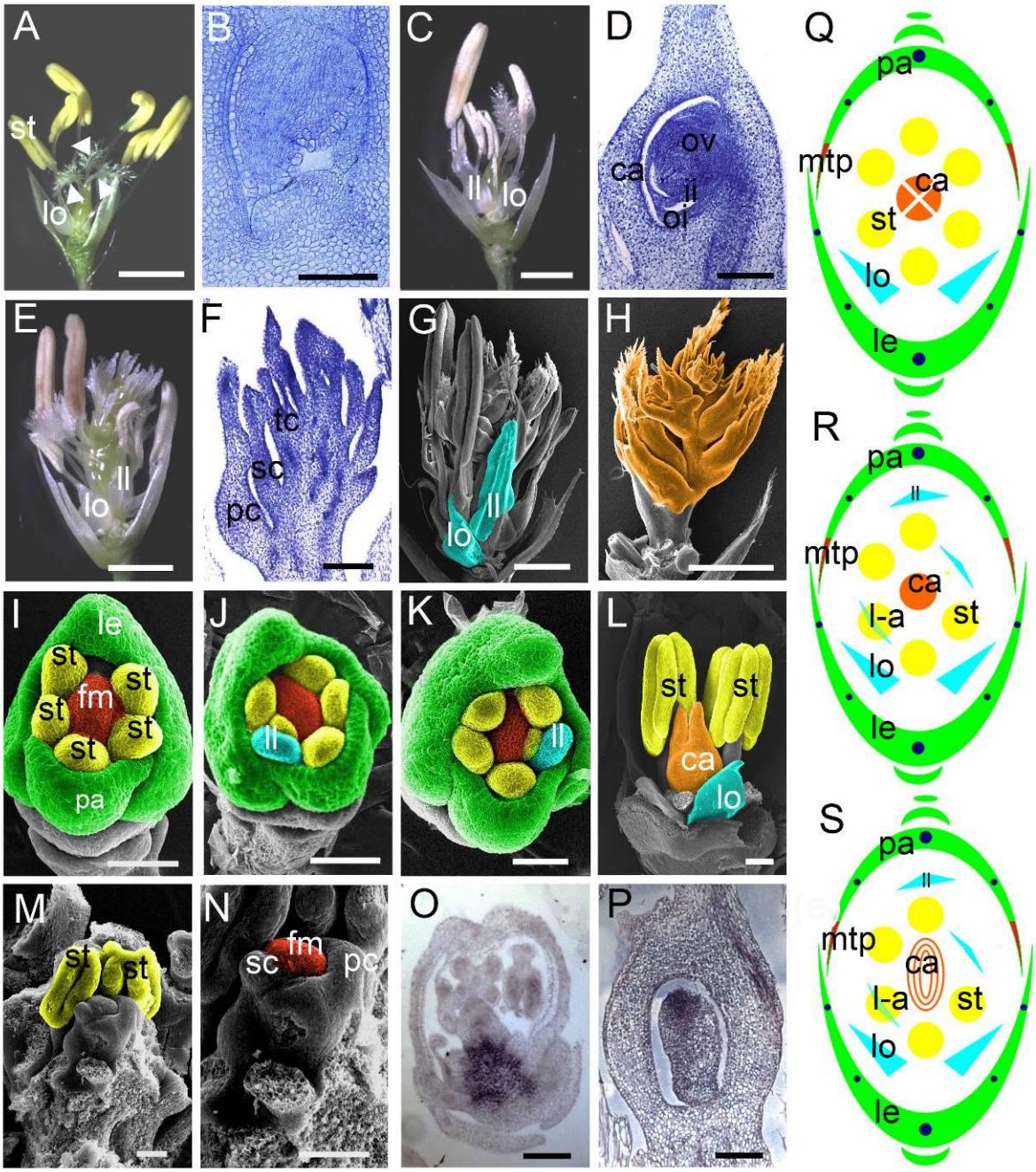
Supplemental Figure 3. OsMADS3 does not interact with OsMADS13 in yeast cells.

The transformants co-transformed with plasmids encoding OsMADS6 and OsMADS13 as the positive control, could grow on the selective medium plate. While the transformants containing both plasmids pGADT7 and pGBKT7 as the negative control, could not grow on the selective medium plate in the same condition. Like the negative control, transformants harboring plasmids encoding OsMADS3 and OsMADS13 could not grow on the selective medium plate.

SD/2- represents the SD medium containing no Trp and Leu, SD/3-/3-AT+ represents the SD medium without His, Trp and Leu, but containing 3-AT with optimized concentration.

Figures and Figure Legends

Fig.1



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C, One *osmads3-4* flower with the removal of the lemma and the palea.

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L, SEM observation of wild type flower at early stage Sp8 showing the termination of the floral meristem activity.

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P, Expression pattern of *OSH1* in *osmads13-3 osmads3-4* flower at stage Sp8.

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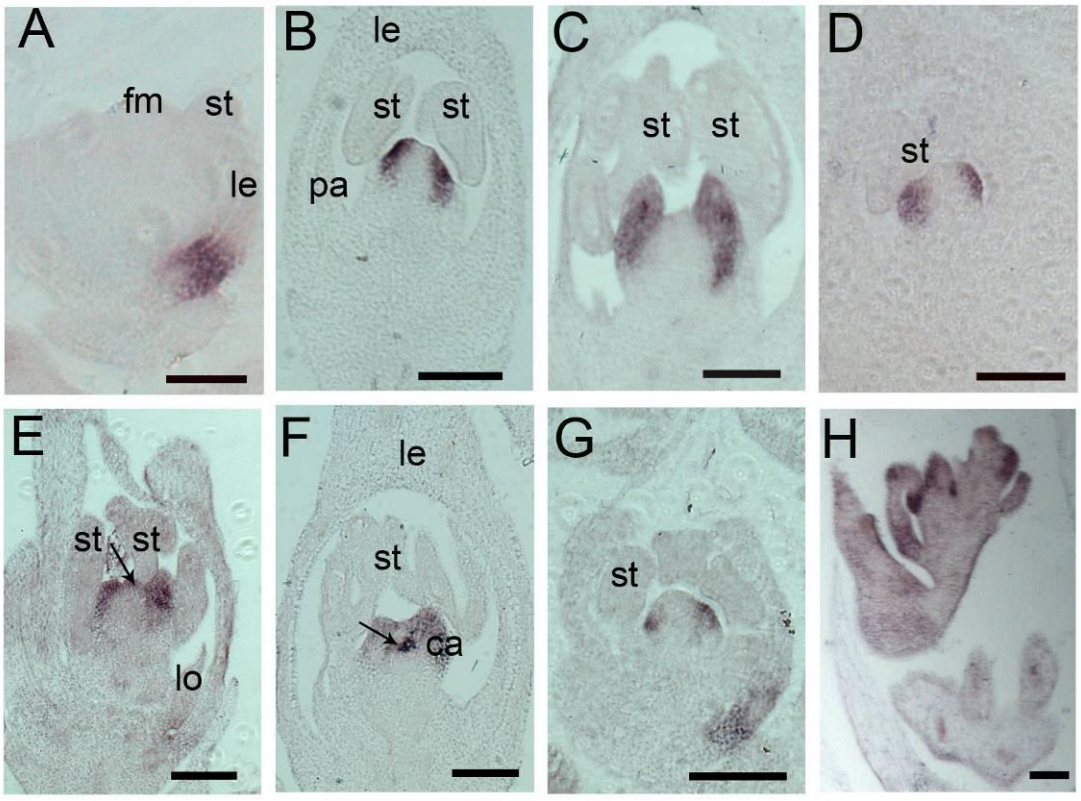

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D-F, *In situ* hybridization of *DL* mRNA in *osmads13-3* flowers.

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G, Normal expression of *DL* in *osmads3-4* at stage Sp7.

H, Ectopic expression of *DL* in *osmads3-4 osmads13-3* at stage Sp8.

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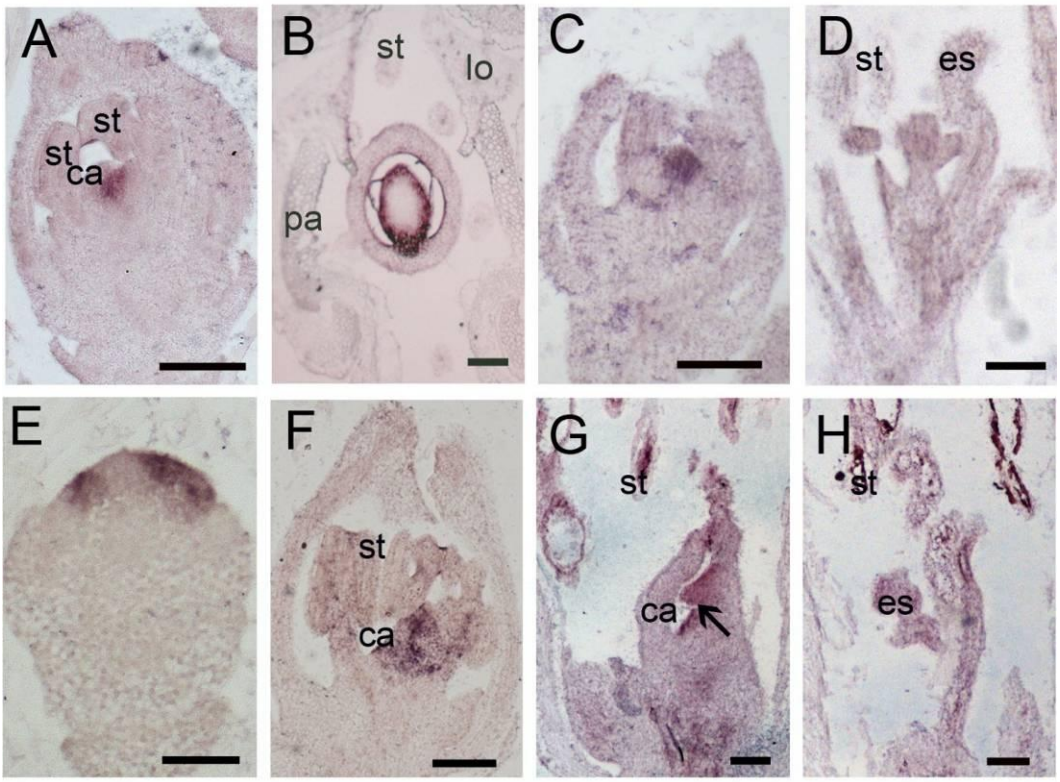
Fig.3



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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, the 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.

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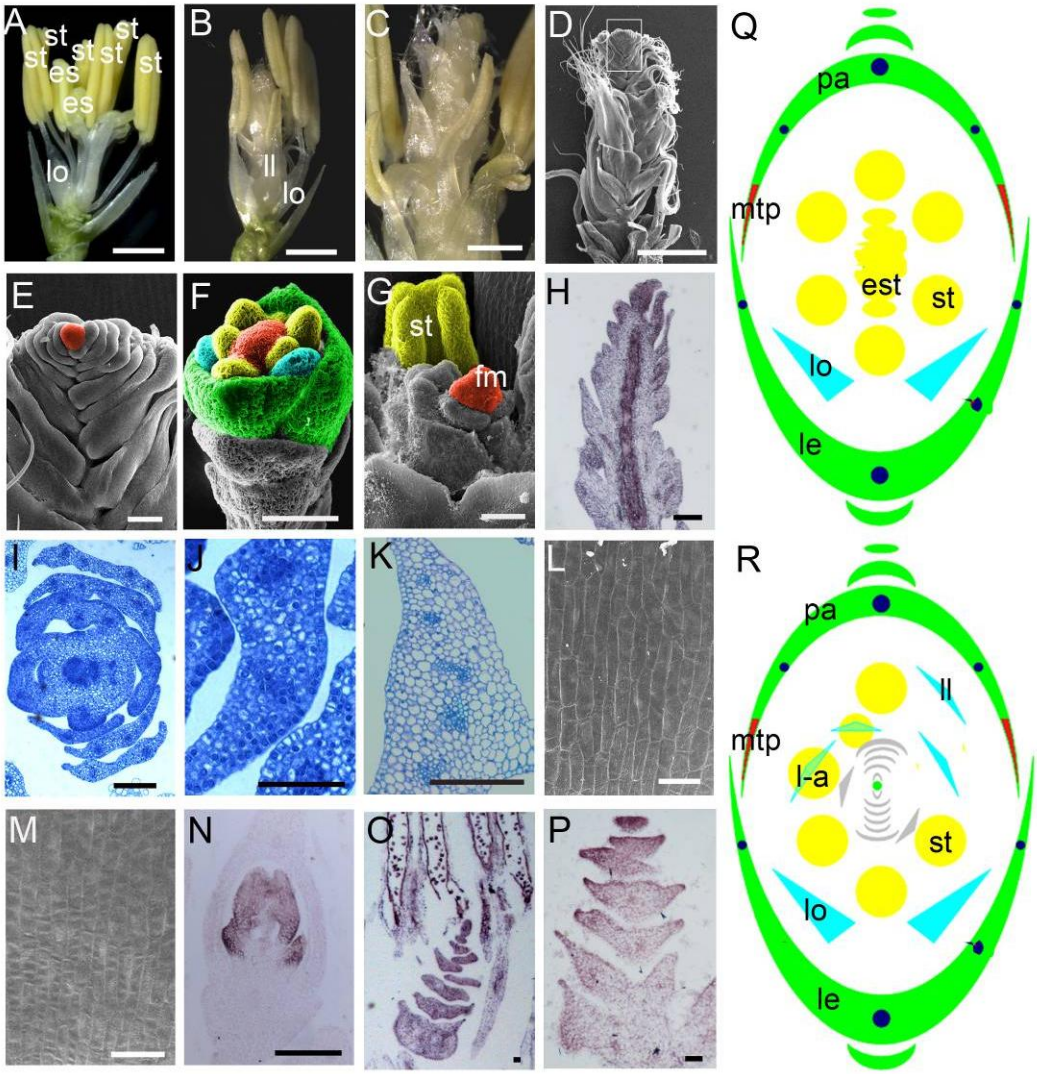
Fig.4



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C, Close-up 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, Close-up of D.

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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 section of one *dl-sup6 osmads3-4* flower showing the ectopic organs in the flower center.

K, Transverse section of one wild-type lodicule.

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Fig.5

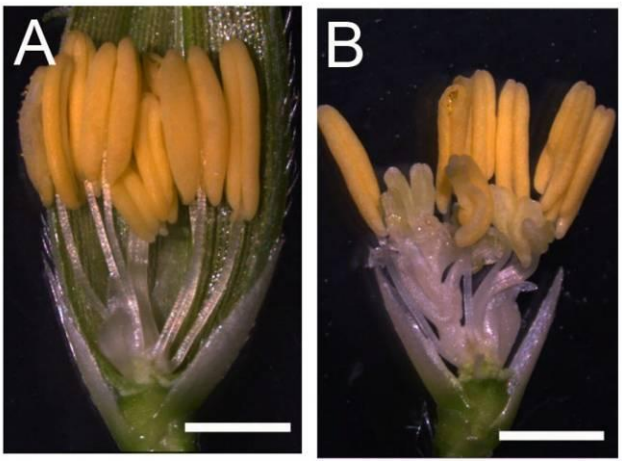

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Fig.6

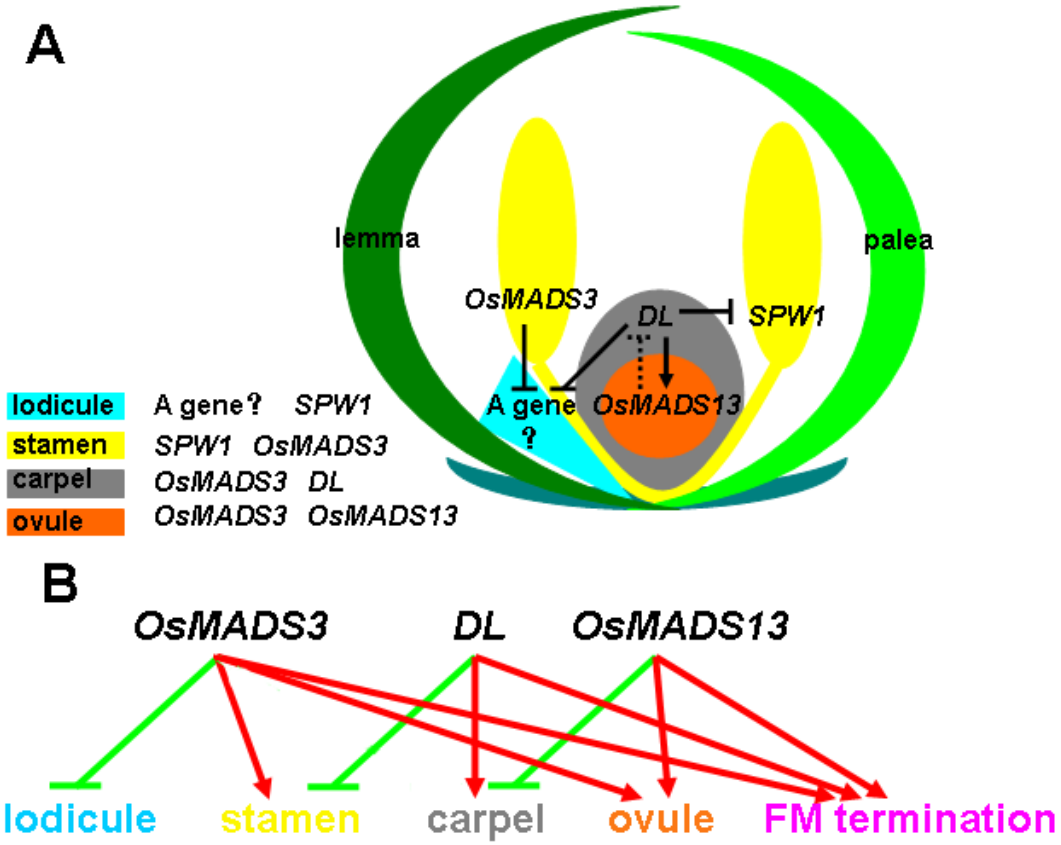


Figure 6. Proposed model to illustrate the genetic interaction between *OsMADS3*, *OsMADS13* and *DL* in rice flower development.

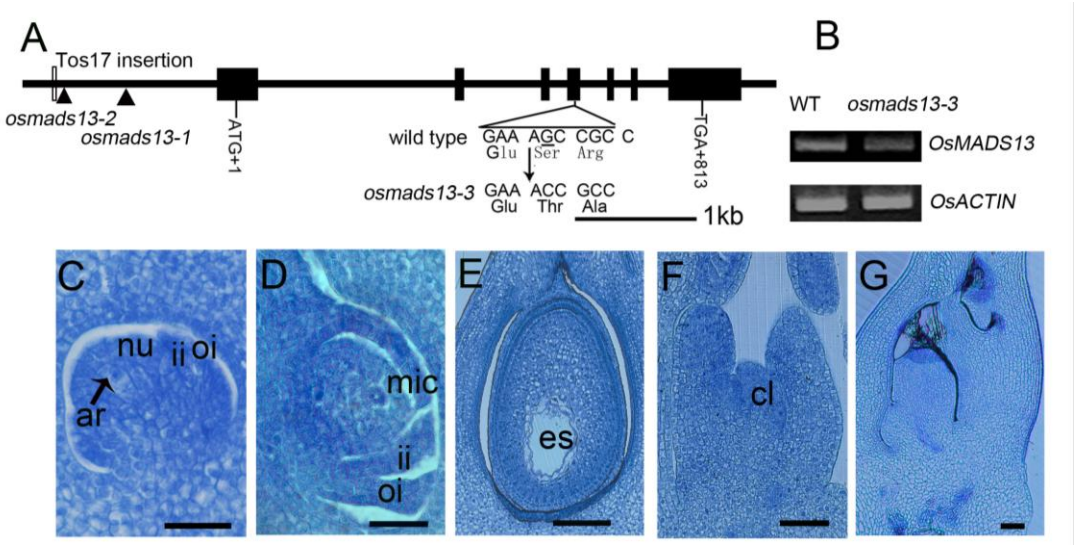
A, Interactions between rice floral organ homeotic genes of A-function gene(s) (such as *OsMADS15*), *SPW1*, *OsMADS3*, *OsMADS13* and *DL*. Different colors represent expression pattern of genes in lodicules, stamens, the carpel and the ovule, respectively. *OsMADS3* possibly represses the expression of A-function gene(s) such as *OsMADS15* in the inner floral organs; *DL* may antagonize the expression of *SPW1* and *OsMADS15* respectively. While *OsMADS13* may indirectly limit the expression of *DL* in the ovule, and *DL* may directly or indirectly positively regulates the *OsMADS13* expression.

B, Functions of *OsMADS3, DL and OsMADS13* in specifying floral organ identities and floral meristem termination. Green lines and red arrows indicate the function of repression and promotion, respectively. Broken arrow means the possibly indirect or

direct regulation of the *OsMADS13* expression by *DL*. *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 determinates the stamen identity in whorl 3, and specifies ovule identity in the floral center, respectively. *DL* represses the formation of stamens and specifies the carpel identity in the flower center, while *OsMADS13* represses the carpel formation and determines the ovule identity. *OsMADS13* may terminate floral meristem termination in parallel with *OsMADS3*, *DL* may regulate the floral meristem determinacy in the same pathway of *OsMADS13*. *OsMADS3* and *DL* can redundantly terminate the floral meristem.

Supplemental Figures

Supplemental Figure 1



Supplemental Figure 1. Schematic representation of *osmads13* mutants and abnormal ovule development of *osmads13* mutants.

A, Schematic representation of *osmads13*.

B, Reduced expression of *OsMADS13* in *osmads13-3.*

C, One wild-type flower at stage Ov2 when the megasporogenesis starts with the formation of the distinctive archespore indicated by the arrow as well as the initiation of outer and inner integument primordia.

D, One wild-type flower at stage Ov4 when the inner integument encloses the nucellus exept for the micropyle.

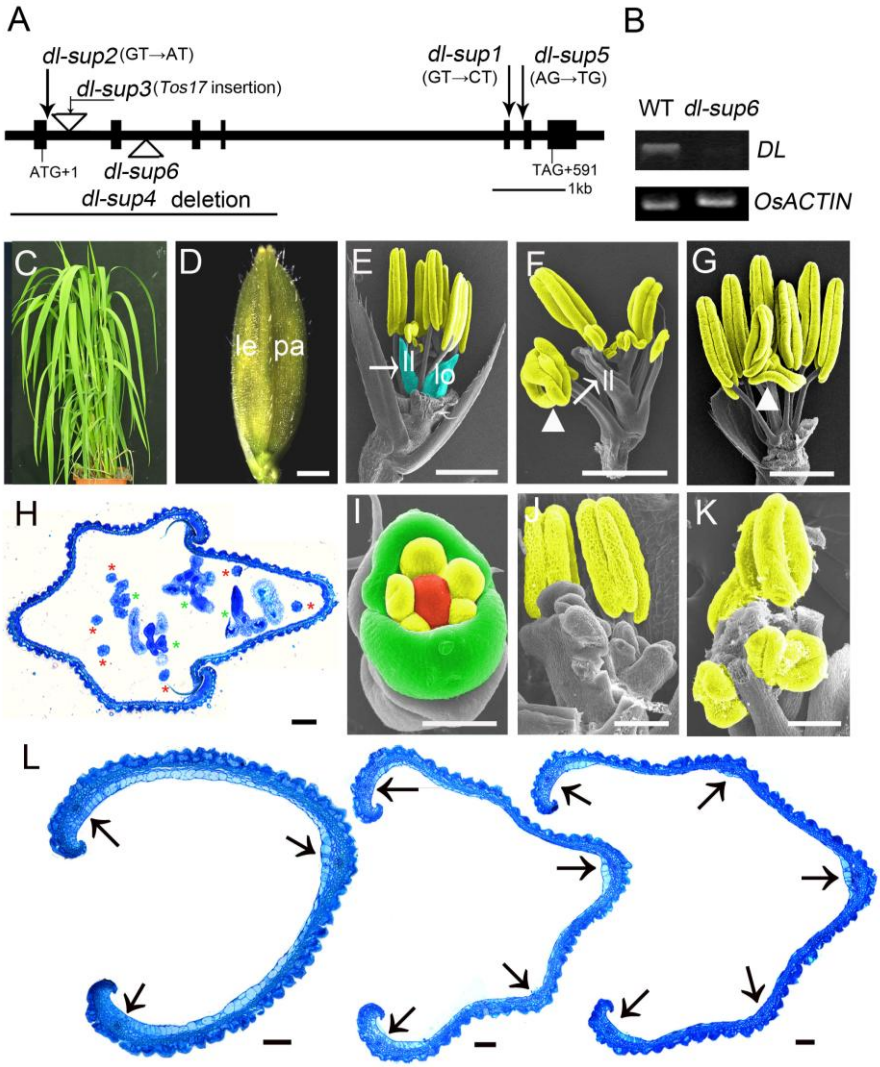
E, One wild-type flower at stage Ov9 when the embryo sac is mature.

F, One *osmads13-3* flower at stage Ov2 displayed the ectopic carpel within the primary carpel.

G, One *osmads13-3* flower at stage Ov9 with indeterminate floral organ at the position of the wild-type ovule.

Ca, carpel; nu, nucellus; ar, archespore; ii, inner integument; oi, outer integument; mic, micropylar pole; es, embryo sac; cl, carpel like structure; ovule developmental stages

refer to Lopez et al (1999).Bars= 200 µm in C to G.



Supplemental Figure 2. Schematic representation of *dl-sup* mutants and phenotype of *dl-sup6*.

A, Schematic representation of *dl-sup* mutants.

B, No detectable *DL* expression in *dl-sup6* leaves.

C, One *dl-sup6* plant displaying drooping leaves.

D, One *dl-sup6* spikelet at stage Sp8.

E, SEM analysis of one *dl-sup6* flower at stage Sp8 with severe floral defects. The lemma and palea were ripped off to show the inner floral organs.

F, One *dl-sup6* flower at stage Sp8 displaying loss of floral determinacy in the floral center revealed by SEM analysis.

G, One *dl-sup6* flower at stage Sp8 with weak floral defects revealed by SEM analysis. The lemma and palea were ripped off to show the inner floral organs. Arrowheads in F and G indicate fused anthers.

H, Transverse section of *dl-sup6* flower to show normal stamens in the third whorl and ectopic organs in the center. Normal stamens and ectopic stamens are indicated by red and green asterisks respectively.

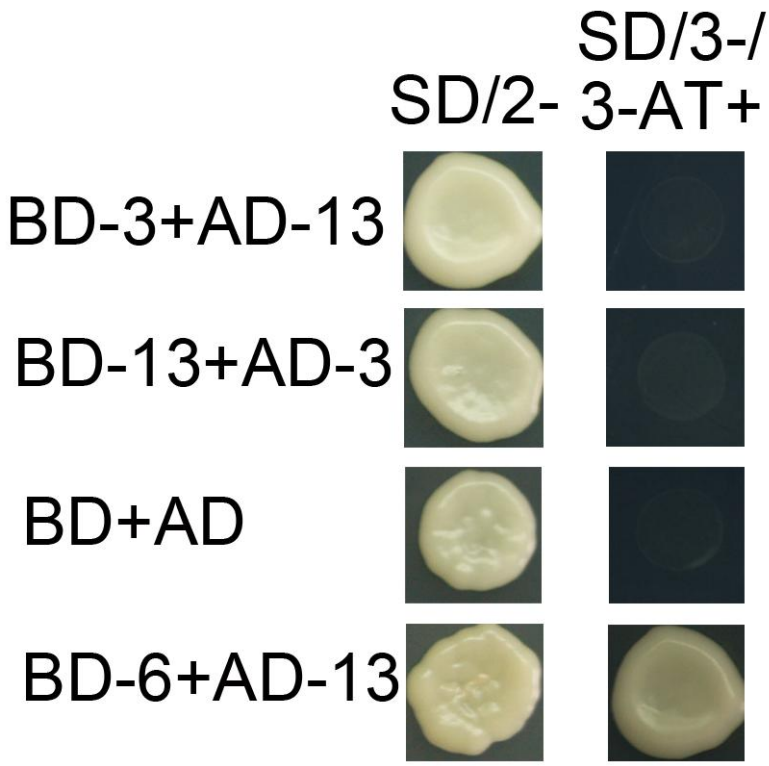
I, SEM analysis revealed normal floral development *dl-sup6* at stage Sp6.

J and K, SEM analysis showed primordia of ectopic organs in *dl-sup6* flowers with strong and weak phenotyps at early stage of Sp8, respectively.

L, Transverse sections of *dl-sup6* showing three (left), four (middle) and five (right) vascular bundles, respectively, in the lemma.

Arrows in E and F indicate the lodicules-like organs in flower center. Le, lemma; pa, palea; ll, lodicules-like organs; lo, lodicules. Bars=1mm in D to G; 100 μm in H, J-L; and 50 μm in I.

Supplemental Figure 3



Supplemental Figure 3. OsMADS3 does not interact with OsMADS13 in yeast cells.

The transformants co-transformed with plasmids encoding OsMADS6 and OsMADS13 as the positive control, could grow on the selective medium plate. While the transformants containing both plasmids pGADT7 and pGBKT7 as the negative control, could not grow on the selective medium plate in the same condition. Like the negative control, transformants harboring plasmids encoding OsMADS3 and OsMADS13 could not grow on the selective medium plate.

SD/2- represents the SD medium containing no Trp and Leu, SD/3-/3-AT+ represents the SD medium without His, Trp and Leu, but containing 3-AT with optimized concentration.

Supplemental Table 1. Primers used in this research

Name	Forward primers (5' → 3')	Name	Reverse primers(5' → 3')
13TPF	AGATGCTGCAAAACACCAACAA	13TPR	TGATCTCTGAAGCCAGCAGTTC
3TPF	ACCAGCAGGAGTCCTCCAAAC	3TPR	CAACTTCAGCATATAACAGCTCATTC
13PPF	GGTGGTGAATTCACTGAAGGAGCTGAAGCAAC	13PPR	GGTGGTGGATCCGGGATTAACAAAATGCAGCAGC
16PPF	GGTGGTGAATTCTACCAGCAAGCCATCGGC	16PPR	GGTGGTGGATCCGCGATGATATATCAACCGAGGC
DLPPF	GGTGGTGAATTCTGTGGCCACTGCAACAACCT	DLPPR	GGTGGTGGATCCTTCACCGCCGATATAGATCG
3YPF	GGTGGTGAATTCAGTGTGAAATCCACCGTTGAGAG	3YPR	GGTGGTGGATCCTAACGTACGTGTGTACGTACGGT
13YPF	GGTGGTCATATGAACAATGTGAAGGCTACAATTGACA	13YPR	GGTGGTGAATTCATGAGGTTCAGAAGTGAGGAGG
6YPF	GGTGGTCATATGGCCGGCATAACAAAGACTTTAGA	6YPR	GGTGGTGAATTCTTGCTGCATGGCTCTGTAGTTG