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Evolution of Developmental Control Mechanisms

Ovule is a lateral organ finally differentiated from the terminating floral meristem in rice

Shinichiro Yamaki [a], Yasuo Nagato [b], Nori Kurata [a,c], Ken-Ichi Nonomura [c,d,*]

[a] Genetic Strain Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan
[b] Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan
[c] School of Life Science, Graduate University for Advanced Studies/SOKENDAI, Mishima, Shizuoka 411-8540, Japan
[d] Experimental Farm, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan

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ABSTRACT

The floral meristem is the homeostatic source of floral organs in angiosperms. In rice, after stamen and carpel differentiation, the floral meristem is terminated and exhausted to produce an ovule. To elucidate underlying mechanisms controlling the continuous process from floral meristem termination to ovule formation, we investigated two rice mutants showing abnormalities in ovule formation. In the weak mutant of the *lonely guy* gene, responsible for cytokinin activation to maintain the floral meristem, ovule formation was abolished inside the normally developing carpel. The loss-of-function of the *OsMADS13* gene, encoding a MADS-box transcription factor, resulted in the replacement of ovule with extra carpels. The *in situ* expression of tissue-specific markers in both mutants revealed that a lateral region of the terminating floral meristem adjacent to the site of carpel initiation exclusively differentiated the ovule and is apparently distinct from the remainder of the floral meristem, in contrast to previous assumptions. Our findings also suggest that primordial germ cells are initiated independently of ovule formation, but dependently on the presence of active cytokinin. We propose a novel pattern of ovule formation in angiosperms, in which the ovule is a lateral organ finally differentiated from the terminating floral meristem in rice.

Introduction

A central issue concerning flower development is to elucidate the regulatory mechanism for the maintenance of the floral meristem (FM). The meristem is an indeterminate proliferative organ, which presents at the growing tips of roots and shoots, and continuously generates new cells postembryonically for organogenesis in plants (Dinneny and Benfey, 2008). After the transition from vegetative to reproductive growth, the FM differentiates nonreproductive organs, the sepal and petal, and reproductive organs, the stamen and pistil. During this process, the FM gradually ceases homeostasis, and is finally terminated and exhausted as pistil founder cells. The pistil is a female reproductive organ composed of carpels and ovules. Primordial germ cells are differentiated at the hypodermis of stamens and ovules.

The pattern of pistil formation is diverse among angiosperm species. In *Arabidopsis thaliana*, the FM is exhausted as a pair of carpels after stamen differentiation. The carpels differentiate a number of ovules at their margins, and fuse with each other to form a single pistil. In petunia, the FM is exhausted as a placenta after stamen and carpel differentiation, and the placenta differentiates many ovules (Angenent and Colombo, 1996; Colombo et al., 2008). In both species, ovule formation is mediated by carpel or placenta, the final products of the FM. In contrast, in rice, a single ovule is the final and direct product of the FM (Yamaguchi et al., 2004; Yamaki et al., 2005). Despite the fundamental process of sexual plant reproduction, no genetic model to comprehensively explain this difference has been proposed.

A key regulator of reproductive organ formation and FM determinacy is the *AGAMOUS* (*AG*) clade genes, encoding MADS-box transcription factors. In the *Arabidopsis ag* mutant, the FM differentiates extra sepals and petals indeterminately in place of stamens and carpels, suggesting that *AG* has two roles, one in establishing the identities of stamen and carpel, and the other in FM termination (Bowman et al., 1989, 1991; Yanofsky et al., 1990). AG protein directly activates genes responsible for various steps of reproductive organogenesis (Savidge et al., 1995; Ito et al., 2004; Gómez-mena et al., 2005; Ito et al., 2007). In addition, AG also activates a zinc-finger transcription factor-encoding gene, *KNUCKLES*, to suppress a homeobox gene *WUSCHEL* (*WUS*), which secures the stem cell niche at the central zone of the FM (Mayer et al., 1998; Payne et al., 2004; Sun et al., 2009). This dual function of AG at the molecular level indicates that reproductive organ formation and FM determinacy are coordinated in an identical regulatory pathway. The dual function is widely

* Corresponding author. Experimental Farm, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan. Fax: +81 55 981 5872.
E-mail address: knonomur@lab.nig.ac.jp (K.-I. Nonomura).

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conserved in *AG* orthologs of various angiosperm species, including petunia and rice (Kater et al., 1998; Kapoor et al., 2002; Kyozuka and Shimamoto, 2002; Yamaguchi et al., 2006).

In contrast to *AG* genes, the *SEEDSTICK*/*FBP11* clade genes, encoding the other AG subfamily MADS-box proteins, are specifically expressed in ovules (Schmidt et al., 1993; Angenent et al., 1995; Rounsley et al., 1995; Lopez-Dee et al., 1999; Yamada et al., 2009; Tani et al., 2009). In *Arabidopsis*, the *SEEDSTICK* (*STK*) gene plays a role in differentiation of carpel margin to ovule founder cells, because the loss-of-function *stk* mutant exhibits homeotic conversion of ovules to secondary ectopic carpels when combined with mutations of paralogous AG clade genes, *SHATTERPROOF1* (*SHP1*) and *SHP2* (Pinyopich et al., 2003). The carpel margin is regarded as a meristematic indeterminate tissue in *Arabidopsis*, because it expresses the *SHOOT MERISTEMLESS* (*STM*) and *KNAT2* genes, members of class 1 KNOX genes conferring the indeterminate feature on meristem cells (Long et al., 1996; Pautot et al., 2001). The inducible overexpression of *STM* or *KNAT2* causes homeotic conversion of ovules to carpels, similarly to the *stk* mutant (Pautot et al., 2001; Scofield et al., 2007). These observations raise a possibility that *STK* establishes the ovule identity by suppressing the indeterminate state of cells in the carpel margin. In petunia, two orthologs of *STK*, *FBP7* and *FBP11*, are also expressed specifically in ovules (Angenent et al., 1995), subsequently to the expression of petunia *AG* orthologs, *pMADS3* and *FBP6* (Kater et al., 1998; Kapoor et al., 2002). The co-suppression of *FBP11* also results in the homeotic conversion of ovules to carpels on the placenta (Angenent et al., 1995). In contrast, ectopic *FBP11* expression results in the formation of ectopic ovules on sepals and petals (Colombo et al., 1995). Taken together, it is plausible that the *STK*/*FBP11* clade genes have a common function to establish ovule identity, distinct from the functions of *AG*/*pMADS3*/*FBP6* clade genes in reproductive organogenesis and FM termination.

In rice, an *STK*/*FBP11* clade gene, *OsMADS13*, is expressed in both the terminating FM and ovule (Lopez-Dee et al., 1999; Yamaki et al., 2005). *OsMADS13* expression is preceded by expression of *OsMADS3* and *OsMADS58*, the rice orthologs of *Arabidopsis AG* (Yamaguchi et al., 2006), indicating that the genetic system controlling floral development in *Arabidopsis* and petunia is largely conserved in rice. Because the loss-of-function *osmads13* mutant forms extra carpels in place of a single ovule, it has been thought that *OsMADS13* regulates FM determinacy in addition to establishing ovule identity (Dreni et al., 2007). However, because the *Arabidopsis stk* mutant and petunia co-suppression transformant of *FBP11* do not show defects in FM determinacy (Colombo et al., 1995; Pinyopich et al., 2003), this assumption about *OsMADS13* function seems to be inconsistent with the *STK*/*FBP11* function specifically in ovule formation. In addition, it has not been clearly shown how FM termination is disturbed and how the ovule is replaced with extra carpels in the *osmads13* mutant. In this study, we will prove that rice *OsMADS13* has a specific role in ovule development, comparable to *Arabidopsis STK* and petunia *FBP11*, from analyses using several rice genes and mutants. This study also indicates that the rice ovule is a lateral organ directly and finally differentiated from the FM, and that FM termination and subsequent germ-cell initiation are orchestrated by a complicated genetic network.

Materials and methods

Plant materials

The *lonely guy-3* (*log-3*) mutant was found from an R_2 population of rice (*Oryza sativa* L.) cv. Nipponbare regenerated from callus suspension culture, described by Kurakawa et al. (2007). The *log-3* mutant has a substitution of guanine to alanine at 24.6 Mb of chromosome 1, which resulted in a premature stop codon at W223 of 242 amino acids in the *LONELY GUY* locus (Fig. S1A). Although 9.7% of *log-3* spikelets lacked stamens, 90.3% of *log-3* spikelets formed stamens successfully. In contrast, 15.8% of *log-3* spikelets lacked pistils and 65.3% of *log-3* spikelets lacked ovules in pistils (see below). Therefore, the *log-3* mutant is mainly regarded as a female sterile mutant.

The *floral organ number1-1* (*fon1-1*) mutant was isolated from an M_2 population of rice cv. Taichung 65 mutagenized with *N*-methyl-*N*-nitrosourea. *fon1-1* has a mutation of cytosine to thymine, which results in the substitution of proline to leucine in the C-terminal Ser/Thr kinase domain of FON1 protein (Suzaki et al., 2004). In the *fon1-1* mutant, the FM was remarkably enlarged after stamen differentiation, and differentiated an increased number of pistils (Nagasawa et al., 1996; Suzaki et al., 2004; see below).

Five lines that had retrotransposon *Tos17* insertions into the *OsMADS13* locus were found from mutant panels of rice cv. Nipponbare (Hirochika et al., 1996; http://tos.nias.affrc.go.jp/). These lines, ND4064, NG4680, NG4927, NG4955 and NG5195, had *Tos17* insertions at three independent positions in the first intron of *OsMADS13* (Fig. S1B). These lines segregated plants with floral abnormalities described below in the manner of a single recessive gene. The abnormality in mature spikelets of these lines was identical to those of the *osmads13* mutant reported by Dreni et al. (2007). In addition, this abnormality was completely linked with *Tos17* insertion homozygous genotypes and with the deformity in the 5′ UTR of *OsMADS13* mRNA (Fig. S1C). Thereafter, we concluded that their phenotype was derived from *Tos17* insertion into the *OsMADS13* locus. In this study, we used the *Tos17* insertion homozygote ND4064 plant as our *osmads13-2* mutant. Nipponbare was used as wild type. Wild-type and mutant plants were grown in pots or a paddy field under natural condition.

Genotyping and RT-PCR analysis

Genotyping of *Tos17* insertions into the *OsMADS13* locus was performed by PCR using a *Tos17*-specific primer (5′-AGGTTGCAAGTTAGTTAAGA-3′) and *OsMADS13*-specific primers (5′-TCACACAGCAGCAGAAAACC-3′ and 5′-GAAGCATCAAACTGGAAGCC-3′ for ND4064, NG4680, NG4927 and NG4955; 5′-TTTTTCGCTTCTTCCCTTCA-3′ and 5′-GAAAGACTTGGCGGATATGG-3′ for NG5195). Structures of *OsMADS13* mRNAs in *Tos17* insertional lines were analyzed by RT-PCR using *OsMADS13*-specific primers (5′-AGAAAACCCACCAACTGGAGCTCTC-3′, 5′-CTGAGCATTGACCTCTATGAGAGG-3′, 5′-CGTATGAGCTCTCCGTCCTC-3′ and 5′-GCCTCGAACAGGTTCAACG-3′; Fig. S2) and by the direct sequencing of RT-PCR products.

Histological analysis and in situ hybridization

Samples were fixed in FAA for 24 h at 4 °C, and then dehydrated in a graded ethanol series. The ethanol was replaced with xylene and the samples were embedded in Paraplast Plus (McCormick Scientific).

Paraffin samples were sectioned using a microtome (HM315; Microm). For histological observation, 8 μm thick sections were applied to microscope slides coated with PLL (Matsunami Glass). Sections were stained with Delafield's hematoxylin (Muto Pure Chemicals). For *in situ* hybridization, 8 μm thick sections for single probe hybridization or 4 μm thick sections for two-probe hybridization using adjacent sections were applied to microscope slides coated with MAS (Matsunami Glass). For the *OSH1* probe, the full-length cDNA was used as a template. For the *OsMADS13*, *LOG* and *MEL1* probes, cDNA fragments were amplified by PCR using primers specific to each gene (5′-AGATTGAGCTTCAGAACGAC-3′ and 5′-AGCCACATCAGTGTCTGTC-3′ for *OsMADS13*; 5′-AGCAAAAGCCTCCCCCTC-3′ and 5′-CGTCCGCCGGTTAGAAGC-3′ for *LOG*; 5′-GCATTGTCTCAAGCAGAGTTAAGGC-3′ and 5′-CCTGAAATCACCAAATACCG-3′ for *MEL1*), and cloned into pCRII vector (Invitrogen). Digoxigenin-labeled antisense and sense riboprobes were generated by transcription with SP6/T7

RNA polymerase and DIG-RNA labeling mix (Roche). *In situ* hybridization and immunological detection of the hybridization signals were performed as described by Kouchi and Hata (1993). Because no signal could be detected for the sense probe experiment for each gene, only the results of antisense probe experiments are shown.

The slides were observed under a light microscope (DP-50; Olympus). For all section images, the right is the lemma side of spikelet, and the left is the palea side of spikelet, as shown in the first panels of the figures. In the results for early floral development, we described the growing tip as a floral meristem (FM) before the carpel encloses it (for reference, see Fig. 1B), and as an ovule primordium or inner organ after the carpel encloses it (Figs. 1C and M).

Results

Cytokinin activating enzyme LOG is required for ovule initiation

The rice spikelet is composed of two rudimentary glumes, two empty glumes, one lemma, one palea, two lodicules, six stamens and one pistil (Fig. 1A). A set of lodicules, stamens and a pistil is regarded as a flower. During floral organogenesis, the FM has been maintained until carpel initiation (Fig. 1B). After the carpel protrudes from the lemma side of the FM, a lateral region of the FM adjacent to the carpel initiation differentiates into the ovule primordium. The carpel becomes enlarged and exhausts the palea side of the FM to enclose the ovule primordium (Fig. 1C). After the carpel completely encloses the ovule, a megaspore mother cell (MMC) develops at the ovule hypodermis, and an integument primordium, which is divided into inner and outer integuments postgenitally, protrudes from the proximal region of ovule primordium (Fig. 1D). Finally, the ovule in the mature pistil differentiates an embryo sac, the female gametophyte (Figs. 1E and F).

To elucidate genetic mechanisms regulating ovule development in rice, we had tried to identify plants showing defects in ovule formation. In an R_2 population regenerated from suspension-cultured rice calli, we found a somaclonal mutation affecting ovule formation. This mutation caused a premature stop codon in the *LONELY GUY* (*LOG*) gene, described as the *log-3* allele in Kurakawa et al. (2007) (Fig. S1). The *LOG* gene functions in the activation pathway of cytokinin, a phytohormone essential for the maintenance of shoot meristem activity (Kurakawa et al., 2007). Most of *log-3* spikelets formed six stamens, and 65.3% of which possessed a slender pistil lacking an ovule (Figs. 1J and K), while 15.8% formed no pistil (see below). In the *log-3* mutant, the volume of the FM dome was normally maintained before carpel protrusion (data not shown), but subsequently failed to expand to the same extent as the wild type (Fig. 1G). This decreased FM volume caused from the smaller number of cells rather than the size of cells (data not shown). Though the carpel developed normally, the ovule founder region did not differentiate any organs (Figs. 1H and I). Thus, the *log-3* mutant is beneficial for the study of ovule development.

To reveal whether the *log-3* ovuleless phenotype was caused by a shortage of the volume of the FM, we examined the expression pattern of the *OSH1* gene, an ortholog of *Arabidopsis STM* (Matsuoka et al., 1993; Sentoku et al., 2000). In the wild type, *OSH1* was expressed in the FM, and downregulated in developing stamens and carpel (Fig. 2A). As the carpel grew, *OSH1* expression became restricted to the apical inside of the FM dome (Figs. 2B and C), and finally vanished from the ovule primordium (Fig. 2D). Even in the *log-3* mutant, *OSH1* was expressed normally until stamen differentiation (data not shown). At the stage of carpel protrusion, however, the area expressing *OSH1* became smaller than that of the wild type because of the decreased FM volume (Fig. 2E), and was restricted laterally in the FM dome (in 6 of 8 pistils) (Fig. 2F), which differed from its central location in the wild type (Fig. 2B). This difference seemed to be caused

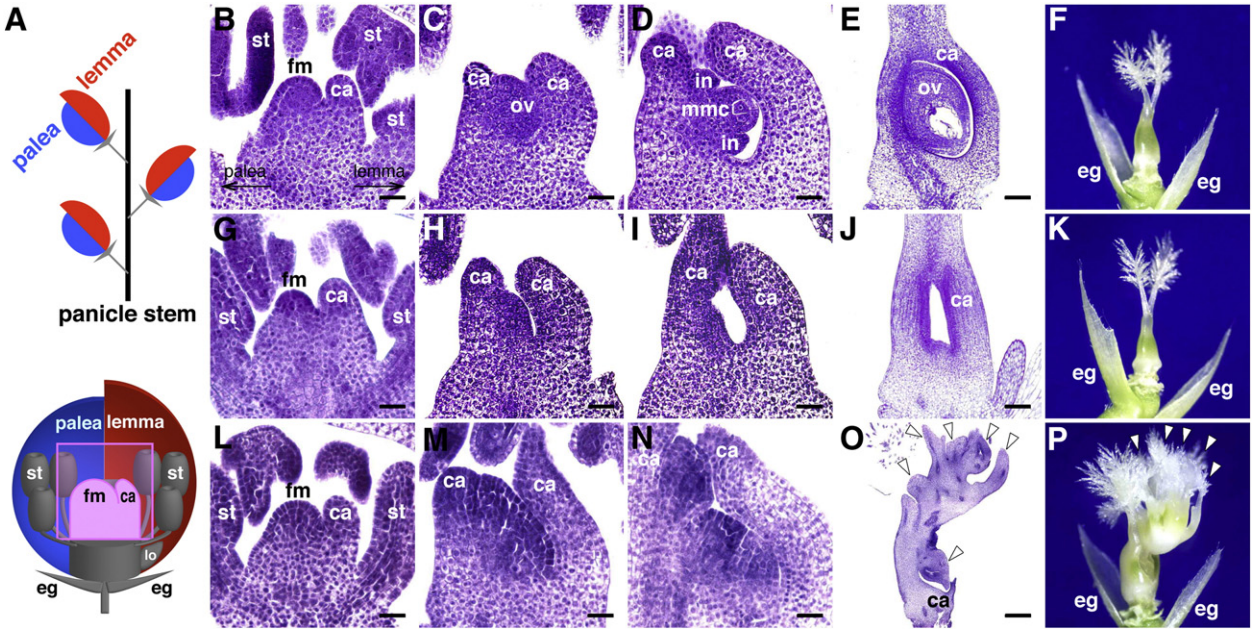


Fig. 1. Phenotypes of *log-3* and *osmads13-2* mutants. (A) Schemes of rice spikelets. Spikelets are arranged in 110° semi-alternate phyllotaxy on panicle stems. The lemma faces the panicle stem in all spikelets. In a spikelet, two rudimentary glumes (not shown), two empty glumes, one lemma, one palea, two lodicules and six stamens are formed. Subsequently, one carpel is differentiated from the lemma side of the floral meristem. The pink plane indicates the orientation of sections in this study. (B–F) Pistil development in the wild type. A carpel protruded from the lemma side of the floral meristem (B), and grew to the palea side with enclosing an ovule primordium (C). When the carpel completely enclosed the ovule primordium, a megaspore mother cell became visible and an integument primordium was differentiated in the ovule primordium (D). In a mature ovule, a vacuolated embryo sac was visible (E). One pistil possesses two stigmas (F). Organs in outer whorls were removed. (G–K) Pistil development in the *log-3* mutant. When a carpel protruded, the floral meristem was smaller than the wild type (G). During carpel growth, the inner organ was still smaller than the wild-type ovule primordium (H), and became indistinguishable from the neighboring carpel (I). The ovule was lost in the mature *log-3* pistil (J). The mature *log-3* pistil was slenderer than the wild type. (L–P) Pistil development in the *osmads13-2* mutant. As the primary carpel grew, the inner organ became irregularly shaped (M), and never exhibited the normal pattern of ovule formation (N). Extra carpels (arrowheads) occupied the inside of the *osmads13-2* primary carpel (O). There was no sign of an embryo sac or integument development. Extra carpels (arrowheads) protruded from the inside of the *osmads13-2* primary carpel. ca, primary carpel. eg, empty glume. fm, floral meristem. in, integument. lo, lodicule. mmc, megaspore mother cell. ov, ovule. st, stamen. Scale bars, 25 μm (C–F, I–L), 100 μm (B), 250 μm (H).

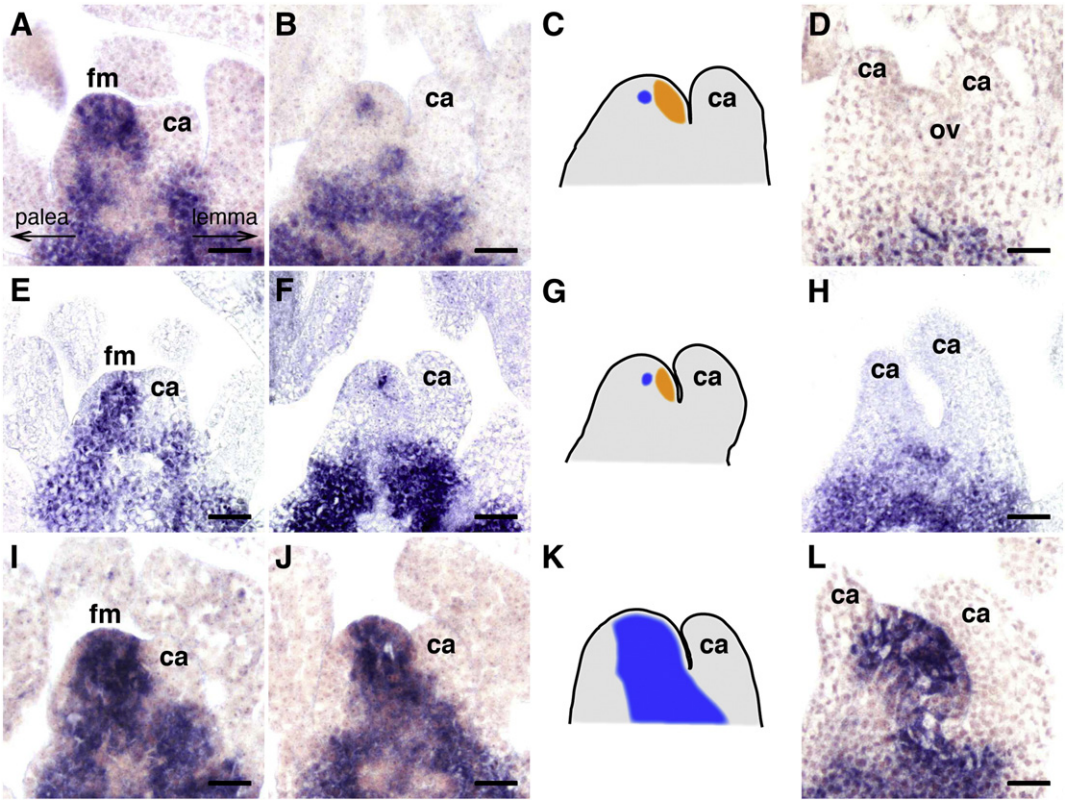


Fig. 2. *OSH1* expression in *log-3* and *osmads13-2* mutants. C, G and K are schematic diagrams of B, F and J, respectively. (A–D) Wild type. In the stage of carpel protrusion from the lemma side, *OSH1* expression was maintained in the floral meristem (A). As the carpel grew, the *OSH1* expression was restricted to the apical part of the floral meristem (B). This restricted *OSH1* expression (C, blue area) visualized the lemma side of the floral meristem (C, orange area). Later, *OSH1* expression vanished from the ovule primordium (D). (E–H) *log-3* mutant. *OSH1* expression normally vanished from the carpel (E), and was restricted to a region of the floral meristem (F). However, the restricted region of *OSH1* expression was closer to the carpel in the floral meristem (G, blue area), and the lemma side of the floral meristem had a smaller volume than the wild type (G, orange area). (I–L) *osmads13-2* mutant. Even after primary carpel protrusion, *OSH1* expression was maintained in the lemma side of the floral meristem (J, K, blue area) and throughout the inner organ (L). ca, carpel. fm, floral meristem. ov, ovule. st, stamen. Scale bars, 25 μm.

by a decreased volume of the lemma side of the FM dome (Fig. 2G), rather than by a dislocation of the *OSH1*-expressing area. According to this interpretation, FM termination, represented by restriction of the *OSH1*-expressing area at the apical inside of the FM dome (Fig. 2B), may not be affected by the *log-3* mutation (Fig. 2F). In subsequent stages, no ovular structure developed in the *log-3* pistil, whereas *OSH1* expression was successfully suppressed as in the wild type (Fig. 2H).

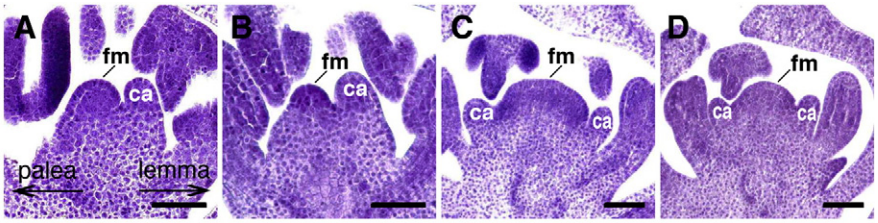
The interpretation that the *log-3* mutation causes a shortage of the FM volume was also supported by the phenotype of the *log-3 fon1-1* double mutant. The single *fon1-1* mutation enlarges the FM size at the stage of carpel protrusion (Nagasawa et al., 1996; Suzaki et al., 2004; Figs. 3A, C and E). In this study, the smaller size of the *log-3* FM was recovered in the *fon1-1* genetic background (Figs. 3B, D and E). In addition, the *log-3* ovuleless phenotype was largely rescued by the *fon1-1* mutation (Fig. 3F). In contrast, the increased number of pistils in the single *fon1-1* mutant was partially suppressed in the *log-3* background (Fig. 3F, Fig. S2).

These results indicate that the function of cytokinin activating enzyme *LOG* is required for ovule formation through maintaining sufficient volume of the FM, in addition for inhibiting the precocious exhaustion of meristems to allow development of normal inflorescence and flowers, as described by Kurakawa et al. (2007). It is also suggested that the lemma side or the lateral region of the FM adjacent to carpel initiation might be a distinct tissue from the remainder of the FM dome.

OsMADS13 is required for ovule initiation from the lemma side of the floral meristem

The *OsMADS13* gene encodes a MADS-box transcription factor required for ovule initiation. Dreni et al. (2007) previously reported that the *osmads13* mutant flower aberrantly differentiates extra carpels, and thus inferred an *OsMADS13* function in FM termination in addition to ovule formation. To verify this assumption, we isolated *osmads13* mutants independently. Five lines with *osmads13* mutations were classified into three allelic groups by the insertion of endogenous retrotransposon *Tos17*, and insertion-homozygous plants were confirmed to express only alternative splicing variants for the first intron of the *OsMADS13* transcript (Fig. S1C). In addition, all these mutations caused essentially the same phenotype as that of the *osmads13* mutant reported previously (Dreni et al., 2007). Thus, we concluded that all lines selected and used in this study were *osmads13* mutants, and show only results from line ND4064, whose mutated allele was designated *osmads13-2* hereafter.

In early development of *osmads13-2* spikelets, the primary carpel was differentiated normally at the lemma side of the FM, and the FM dome was comparable to that of the wild type in shape and size (Fig. 1L). Soon after, however, the ovule founder region became disorganized (Fig. 1M), and ovule pattern formation and MMC differentiation were completely disrupted (Fig. 1N). This disorganized tissue resulted in the aberrant differentiation of plural secondary carpels inside the normally developed primary carpel (Figs. 1O and P). The *DL* gene, which is required for the establishment of carpel identity (Yamaguchi et al., 2004) (Fig. S3A), was expressed in the developing carpel, but neither in the ovule nor any organs inside the pistil in the wild type (Fig. S3A). In contrast, in the *osmads13* pistils, the disorganized tissue ectopically expressed *DL* mRNA laterally in an alternate phyllotaxy (Fig. S3B), indicating that this tissue has FM potential. We also examined the *in situ* expression of *OSH1*. Even in the *osmads13-2* pistil, *OSH1* was expressed normally in the FM before carpel protrusion (Fig. 2I). Subsequently, however, *OSH1* expression



E

	Wild type	*log-3*	*fon1-1*	*log-3 fon1-1*
fm width (μm)	48.2 ± 6.7	42.7 ± 7.3	86.8 ± 22.3	71.9 ± 16.1
fm height (μm)	22.9 ± 7.4	19.9 ± 8.1	31.6 ± 9.8	24.8 ± 6.8
fm height/width	0.47 ± 0.10	0.46 ± 0.12	0.37 ± 0.08	0.34 ± 0.09

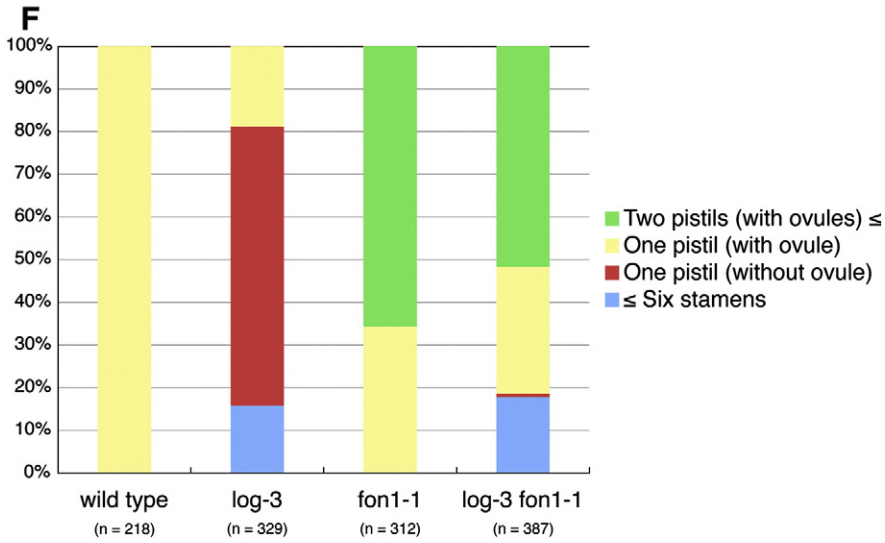


Fig. 3. Rescue of *log-3* ovuleless phenotype by *fon1-1* mutation. (A–D) Floral meristem at the stage after the carpel protrusion. (A) Wild-type floral meristem. (B) *log-3* floral meristem, which was smaller than the wild type. (C) *fon1-1* floral meristem, which was larger than the wild type. Two carpels protruded from the lemma and palea sides of the floral meristem. (D) *log-3 fon1-1* floral meristem, which was larger than the wild type. Two carpels protruded from the lemma and palea sides of the floral meristem, similarly to *fon1-1*. (E) Size of the floral meristem. A small *log-3* floral meristem was recovered in the *fon1-1* background. (F) Frequencies of the innermost organs of mature spikelets. Presence of ovule inside the pistil was investigated by hand dissection. Above 60% of *log-3* spikelets had one pistil without an ovule. *fon1-1* spikelets had one or more pistils with well-developed ovules. In *log-3 fon1-1* spieklets, if pistils formed, almost all of them possessed well-developed ovules. ca, carpel. fm, floral meristem. Scale bars, 50 μm.

continued aberrantly in the lemma side of the FM, in which it is downregulated in the wild type (in all of 15 pistils) (Figs. 2J–L). In addition to the observation of *log-3* pistils, in which a lateral part of the FM was essential to initiate the ovule (Figs. 2E–H), the analysis of *osmads13* pistils demonstrates that *OsMADS13* is required for ovule differentiation from the lateral region of the FM.

Spatiotemporal orchestration of OSH1, OsMADS13, LOG and MEL1 expression during ovule initiation

The *in situ* expression area of *OSH1* seems to be complementary to that of *OsMADS13* (Lopez-Dee et al., 1999; Yamaki et al., 2005). This complementary pattern was determined more precisely in this study using two adjacent sections of a single floral bud for the *in situ* hybridization of two genes (Figs. 4A–J). Before carpel protrusion, *OSH1* was expressed throughout the FM (Figs. 4A and B). In the adjacent section, *OsMADS13* expression had not been initiated yet (Figs. 4F and G). After carpel protrusion, the downregulation of *OSH1* expression at the lemma side preceded its expression in the remaining FM (Fig. 4C), while *OsMADS13* expression was complementarily upregulated in a broader area at the lemma side (Fig. 4H). As the carpel grew, *OSH1* expression was restricted to the apical part of the FM dome, and *OsMADS13* expression had expanded (Figs. 4D and I). When *OSH1* expression disappeared in the ovule primordium, *OsMADS13* expression covered the entire ovule primordium (Figs. 4E and J). In all stages observed, *OsMADS13* expression slightly preceded *OSH1* downregulation.

Next, we compared the expression patterns of *LOG* and *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*) between two adjacent sections (Figs. 4K–T). *MEL1* encodes an ARGONAUTE family protein specifically acting to promote division of premeiotic and meiotic germ cells in both male and female organs (Nonomura et al., 2007), and it is a good marker to observe germ cell lineage. Just after stamen differentiation, *LOG* expression, which is required for the maintenance of the inflorescence and floral meristems, disappeared from the FM tip (Figs. 4K and L), followed by *OSH1* downregulation (Figs. 4B–E). Unexpectedly, after the carpel protruded, *LOG* expression briefly reappeared at the lemma side of the FM (Figs. 4M and N). This *LOG* reappearance was clearly distinguishable from its former appearance at the central tip of the FM dome. This lemma-side, lateral expression of *LOG* was again downregulated during the differentiation of ovule primordium (Fig. 4O). In contrast to *LOG* expression, no *MEL1* expression was detected in the FM dome before carpel protrusion (Figs. 4P and Q). After the carpel protruded, *MEL1* began to be expressed at the hypodermis of the lemma side of the FM (Figs. 4R and S), following and just beneath the laterally reappearing *LOG* expression at the epidermis (Figs. 4M and N). As the carpel grew, *MEL1* expression expanded at the lemma side of the FM and ovule primordium (Fig. 4T). This expanded *MEL1* area indicates that plural primordial germ cells are initiated, while finally restricted to a single MMC during the maturation of the ovule, as described by Nonomura et al. (2007).

These results suggest that the expression of *OSH1*, *OsMADS13*, *LOG* and *MEL1* genes is strictly orchestrated to replace indeterminate cells

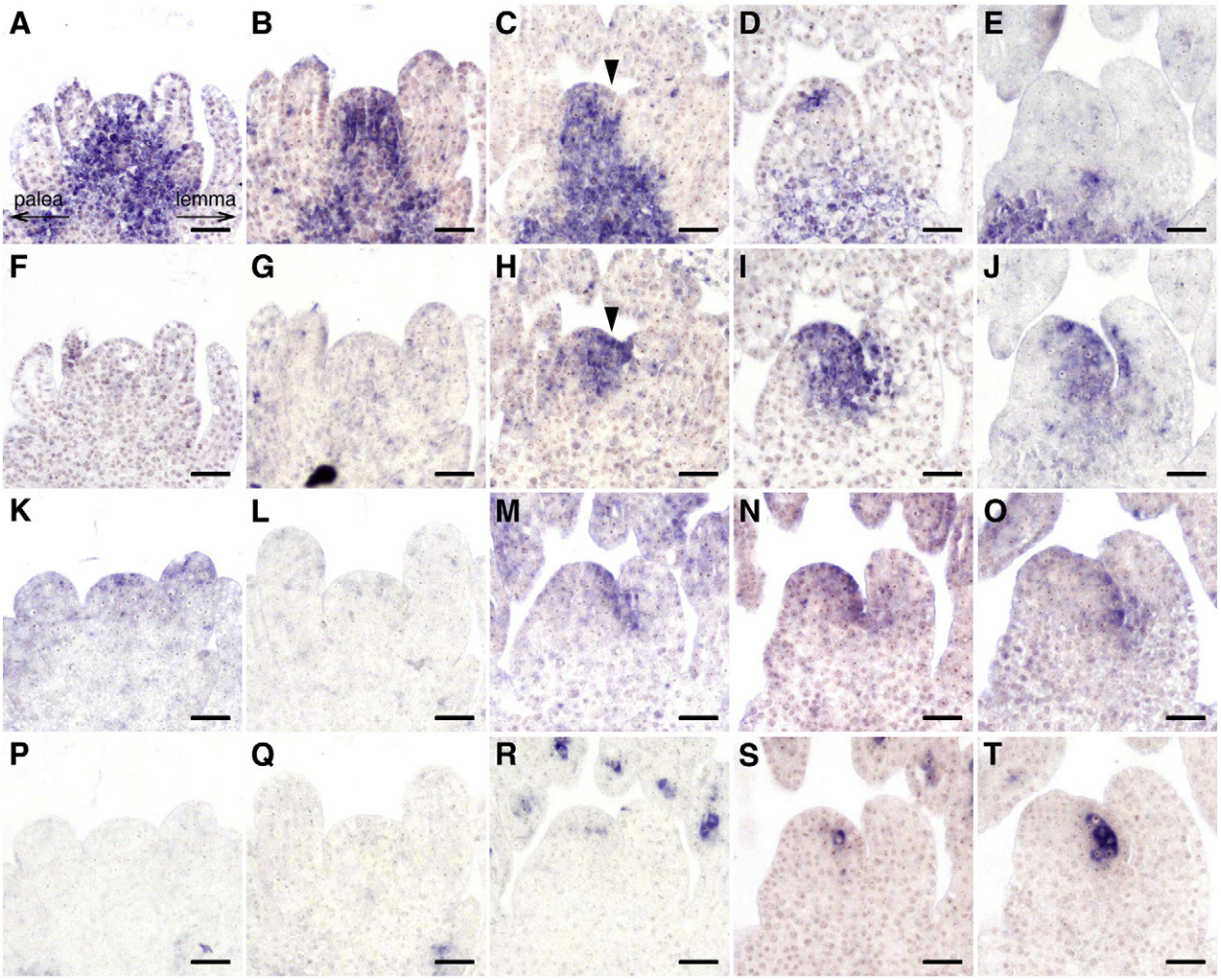


Fig. 4. Comparison of gene expression patterns using adjacent wild-type sections. (A–E) *OSH1* expression. (F–J) *OsMADS13* expression. F, G, H, I and J are adjacent sections to A, B, C, D and E, respectively. *OSH1* was expressed throughout the floral meristem, but not in the floral organ primordia (A and B). After carpel protrusion, *OSH1* expression was downregulated from the lemma side of the floral meristem (arrowhead in C), and *OsMADS13* expression also started from the lemma side of the floral meristem (arrowhead in H). As the *OSH1* expression was restricted to the apical region of the floral meristem, the *OsMADS13* expression expanded throughout the lemma side of the floral meristem, overlapping with *OSH1* expression (D and I). (K–O) *LOG* expression. (P–T) *MEL1* expression. P, Q, R, S and T are adjacent sections to K, L, M, N and O, respectively. *LOG* was expressed at the tip of the floral meristem after stamen differentiation (K). Before carpel protrusion, *LOG* expression once disappeared from the floral meristem (L). Soon after, however, *LOG* expression reappeared in the lemma side of the floral meristem, and was especially strong in epidermal cells (M and N). After *LOG* expression reappeared, *MEL1* expression started and expanded to hypodermal cells beneath the *LOG*-expressing epidermis in the lemma side of the floral meristem (S and T). Subsequently, *LOG* expression became weaker and almost disappeared from the ovule primordium (O). Scale bars, 25 μm.

with an ovule at the lemma side of the FM dome, and that the hypodermal *MEL1* expression may depend on the epidermal *LOG* expression.

Initiation of MEL1 expression is independent of ovule development

One of the next important questions is whether the initiation of primordial germ cells depends on ovule development. To answer this, we investigated the *in situ* expression of the *LOG* and *MEL1* genes in the *osmads13* genetic background. Even in *osmads13-2* flowers, *LOG* expression was normally detected at the central tip of the FM (Fig. 5A), disappeared before primary carpel protrusion (Fig. 5B), and temporarily reappeared at the lemma side of the FM epidermis (Figs. 5C and D). Subsequently, however, *LOG* expression continued aberrantly in the *osmads13-2* pistil (in 15 of 17 pistils) (Fig. 5E), in contrast to its downregulation in the wild type (Fig. 4O). Surprisingly, in spite of the complete absence of ovule formation in the mutant pistil (Figs. 1N and O), *MEL1* expression was initiated just beneath the *LOG*-expressing epidermis as in the wild type (Figs. 5H and I), but failed to be maintained in the disorganized FM tissue inside the primary carpel (in all of 6 pistils) (Fig. 5J). This result indicates that the *OsMADS13* function is independent of the initiation of *MEL1* expression, but is required for the continuous expression of the *MEL1* gene. It also suggests that the epidermal reappearance of *LOG* expression at the lemma side of the FM is important for inducing *MEL1* expression, but that the aberrantly prolonged *LOG* expression may be harmful to stable *MEL1* expression.

We observed the *in situ* expression of *MEL1* and *OsMADS13* genes in the *log-3* ovuleless pistil, because we expected that the lateral *LOG* reappearance would function specifically in primordial germ-cell initiation, in induction of *OsMADS13* expression, or both. In contrast to the expectations, *OsMADS13* and *MEL1* were expressed even in the *log-3* mutant, whereas the expression area became much narrower than in the wild type (Figs. 6A, B). However, it was difficult to reject a function of *LOG* reappearance in the induction of both genes, because its reappearance was also observed in the *log-3* FM (Fig. 6C).

Discussion

Ovule is the final lateral product of floral meristem in rice

This study clearly elucidates that the lemma-side or lateral region of the FM adjacent to the site of carpel initiation is distinct from the

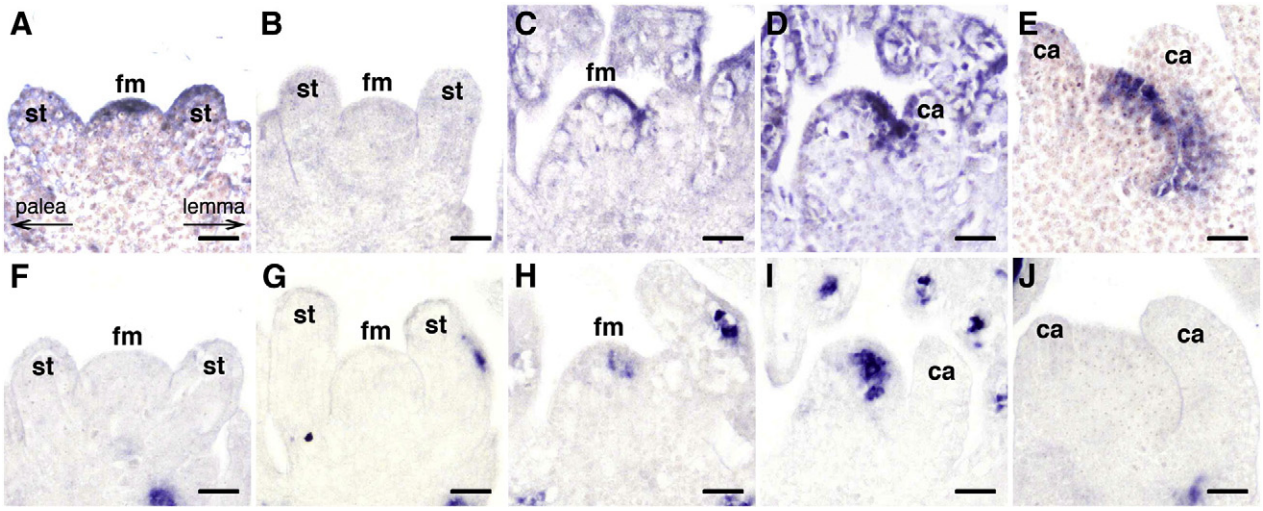


Fig. 5. *LOG* and *MEL1* expression in the *osmads13-2* mutant. (A–E) *LOG* expression. *LOG* expression normally disappeared and reappeared in the lemma side of the floral meristem (B and C). Its expression was also maintained for a prolonged time in the ectopic floral meristem (E). (F–J) *MEL1* expression. *MEL1* expression started and expanded normally in the lemma side of the floral meristem (H and I), but it completely disappeared from the ectopic floral meristem (J). ca, carpel. fm, floral meristem. ov, ovule. st, stamen. Scale bars, 25 μm.

remainder of the terminating FM dome in rice. We call the lateral region of the FM the ovule founder region (OFR) hereafter. The OFR corresponds well to the region where *OSH1* starts to be down-regulated and *OsMADS13* to be upregulated after carpel protrusion (Fig. 4). The loss of function of *OsMADS13* disturbs the normal differentiation of OFR into ovule (Figs. 1 and 2). The resultant disorganized FM in the *osmads13* pistil would be qualitatively equivalent to the indeterminate FM in spikelets of the single *apo1* and double *gym fon2* mutants, because the FM of these mutants continuously differentates extra carpels in an alternate phyllotaxy like that of the *osmads13* mutant (Ikeda et al., 2005, 2007; Yamaki et al., 2005; Fig. S4). However, there are essential differences between *osmads13* and both *apo1* and *gym fon2*. One is that the indeterminate FM of *osmads13* is enclosed with normally developed primary carpel, whereas those of *apo1* and *gym fon2* are not enclosed (Fig. 1; Fig. S4). Another is that the central axis of the indeterminate FM of *osmads13* inclines to the lemma side inside the primary carpel, whereas those of *apo1* and *gym fon2* indeterminate FMs are directed toward the tip of spikelet, without inclining to the lemma side (Fig. 1; Fig. S4). The incline of the primary carpel inner organ represents that the ovule gradually bends during its normal development, clearly indicating that the disorganized FM inside the *osmads13* carpel is derived from the OFR and is formed after normal and regular processes of carpel formation and FM termination. In contrast, in the *apo1* and *gym fon2* mutants, the axis of the indeterminate FM coincides with that of the FM differentiating floral organs except for the ovule, indicating that FM termination is directly disrupted in these mutants. In other words, we propose that the rice ovule originates directly from the FM as a final lateral product. According to this idea, *OsMADS13* would play an essential role in differentiation of OFR indeterminate cells into ovular cells, but not in FM determinacy. This assumption about *OsMADS13* function is coincident with the function of its orthologous *STK*/*FBP11* clade genes in *Arabidopsis* and petunia, and is distinct from the proposal of Dreni et al. (2007) that *OsMADS13* functions in both FM determinacy and ovule formation.

In the typical floral patterning of angiosperms, which differs from rice, the FM is completely exhausted to produce carpel cells, and ovules are formed on the carpel. For example, in *Arabidopsis*, the FM is exhausted as a pair of carpels, and a number of ovules protrude from the carpel margin. In this case, ovule formation is separable from the terminating FM (Fig. 7). Moreover, in *Arabidopsis* mutants in which sepals, petals or stamens are homeotically converted to carpelloid structures, ovules are formed on the margins of these structures (Bowman et al., 1989, 1991; Drews et al., 1991; Jack et al., 1992; Mizukami and Ma, 1992). Therefore in *Arabidopsis*, the ovule is regarded as a part of the carpel. In petunia, after carpel differentiation, the FM is exhausted to produce a placenta, where ovule primordia arise (Angenent and Colombo, 1996; Colombo et al., 2008) (Fig. 7). Because co-suppression of *FBP11*, a petunia ortholog of *OsMADS13*, results in the homeotic conversion of ovule to carpel, rather than to meristem (Angenent et al., 1995), the placenta seems to have a carpel identity in petunia. In addition, in a petunia transformant exhibiting ectopic carpelloidy in stamens, a number of ovules are formed on these carpelloid stamens (Angenent et al., 1993). Therefore, in petunia also, the ovule seems to be a part of the carpel. In contrast, ectopic carpels do not form any ovules in rice mutants in which stamens are

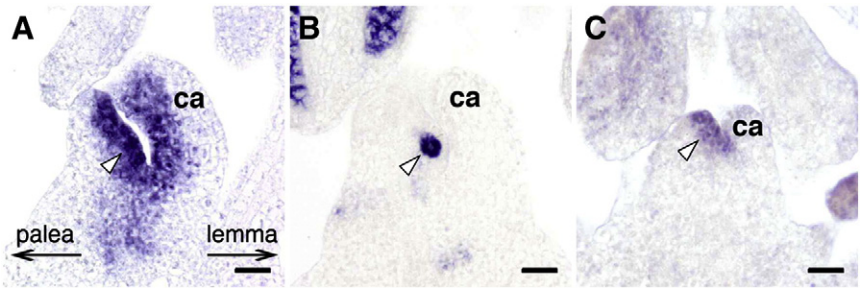


Fig. 6. Gene expression in the *log-3* mutant. (A) *OsMADS13* expression. The *OsMADS13* expression was detected in the small inner organ (arrowhead). (B) *MEL1* expression. Although *MEL1* was expressed in the hypodermis of the inner organ (arrowhead), its expression was detected in a smaller region than the wild type. (C) *LOG* expression. Mutated *LOG* mRNA reappeared as in the wild type. ca, carpel. Scale bars, 25 μm.

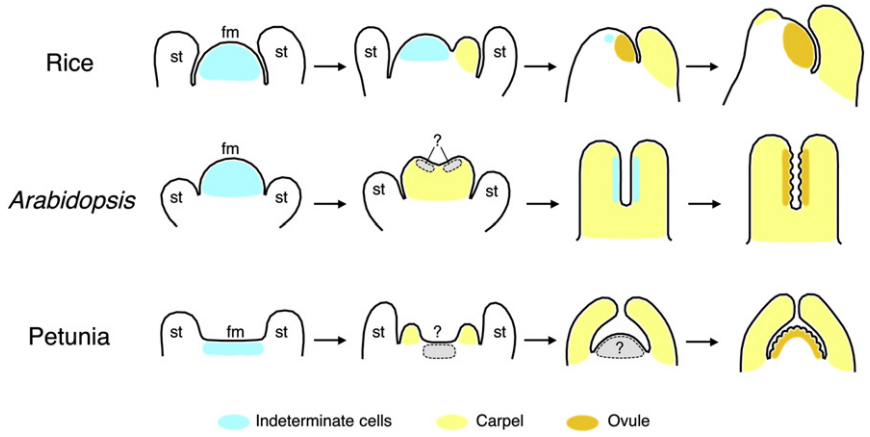


Fig. 7. Schemes of the diversity of ovule differentiation patterns in model plants of angiosperms. In rice, according to our results, the ovule originates from the floral meristem, separately from the carpel cell lineage. Indeterminate cells in the lemma side of the floral meristem are exhausted as ovule founder cells by *OsMADS13* function. In *Arabidopsis*, the floral meristem is exhausted as a pair of carpels. At the stage of carpel growth, indeterminate cells exist at the margin of the carpel, whereas it is unclear whether these cells survived from the floral meristem. Ovules originate from these indeterminate cells at the margin of the carpel by the function of *STK*. In petunia, the floral meristem is exhausted as a placenta. And ovules protrude from the surface of the placenta by the functions of *FBP7* and *FBP11*. It is still unclear how the indeterminate state of floral meristem cells is replaced by ovule identity in the placenta.

homeotically converted to carpels (Nagasawa et al., 2003). In addition, our study indicates that the ovule originates directly from the FM as a lateral organ in rice (Fig. 7). Therefore, the pattern of ovule differentiation is diverse in angiosperms. The direct differentiation of ovule from the FM has been reported in angiosperms for the first time in this study.

Possible relationship between initiation of primordial germline cells and LOG function

One of the important findings of this study is that the expression of the *LOG* gene, which encodes the cytokinin-activating enzyme and is expressed at the epidermal tip of the meristem, once disappears during FM termination, and transiently reappears at the epidermis of the OFR (Fig. 4). From this finding, the simplest explanation for the ovuleless *log-3* phenotype would be that the laterally reappearing *LOG* is required for maintaining an appropriate volume of the OFR to differentiate the ovule primordium (Fig. 2). However, because the *log-3* mutation affects not only the OFR volume, but also the total volume of the FM dome during carpel protrusion (Figs. 1 and 3), the shortage of the OFR volume in the mutant can be fully explained by the previously proposed *LOG* function, which maintains the FM activity (Kurakawa et al., 2007). So, what is the role of the laterally and transiently reappearing *LOG* in the OFR? It is difficult to definite the role from the results of this study, because *LOG* expression reappears even in the *log-3* mutant (Fig. 6), and because severer *log* mutations result in the precocious FM termination until stamen primordium differentiation (Kurakawa et al., 2007). However, it is noteworthy that *MEL1* expression, encoding a germ-cell-specific ARGONAUTE family protein (Nonomura et al., 2007), is induced just beneath epidermally reappearing *LOG* expression (Fig. 4). This result suggests that the *LOG* reappearance may be required for the initiation of *MEL1* expression through secreting active cytokinin from the OFR epidermis. Actually, many sequences potentially bound with proteins responsible for the cytokinin signalling pathway were found in the upstream *cis* region of the *MEL1* gene (K.I.N., unpubl. obs.).

In the rice *mel1* mutant, germ cell development is arrested at early meiosis in male and female reproductive organs (Nonomura et al., 2007). *MEL1* function might be required for the insurance of the proper cell division of germ cells, but not for their initiation, because germ cell lineages seem to develop normally until premeiosis in the *mel1* mutant (Nonomura et al., 2007). In our observations, intriguingly, even under the loss of ovule identity in the *osmads13* pistil, *MEL1* expression and the brief *LOG* reappearance were normally initiated and expanded in the ectopic FM (Fig. 5). This result suggests again a close relationship between the initiation of *MEL1* expression and the brief reappearance of *LOG*. In later stages, however, *MEL1* expression suddenly disappeared, and instead, *LOG* expression was prolonged in the ectopic FM in *osmads13* pistils (Fig. 5). This indicates that while the initiation of *MEL1* expression is independent of the proper ovule or female germ cell development, its stable and continuous expression is dependent on it. Transient and lateral reappearance of *LOG* during FM termination might be required for the initiation of *MEL1* expression, because the *MEL1*-expressing area was significantly reduced by the *log-3* mutation (Fig. 6B). In this context, the coexistence of a meristematic indeterminate feature (ectopic *OSH1* expression) and cytokinin activity (transient *LOG* expression) may trigger the establishment of persistent meristem activity, which in turn eliminates female germ cell development (Fig. S4). To verify these possibilities, further analyses should be done of the function of lateral *LOG* reappearance.

Though there is less information on the transition of somatic to germline cell lineages in plants, another candidate factor responsible for the female germline cell initiation is the *AG* clade genes, *OsMADS3* and *OsMADS58* in rice. In *Arabidopsis*, *AG* directly activates the downstream genes to progress both male and female reproductive organ development (Savidge et al., 1995; Ito et al., 2004, 2007; Gómez-mena et al., 2005). The relationship of *AG* clade genes to the genes responsible for *LOG* reappearance and primordial germ cell initiation remains to be investigated.

Evolutionary aspect of ovule differentiation

This study reveals that the FM is exhausted to produce the ovule as a lateral floral organ in rice. In ovules of higher vascular plants, including angiosperms, the nucellus is regarded as an organ homologous to a megasporangium of early vascular plants (Gifford and Foster, 1989; Stewart and Rothwell, 1993). In the earliest vascular plants, for example Rhyniophyta and Trimerophytophyta, which have homomorphic sporangia, all sporangia are generated at the distal end of bifurcating shoots, which do not possess any lateral organs (Edwards, 1986; Gifford and Foster, 1989). In the shoot architecture of these earliest vascular plants, the sporangium is presumably differentiated apically, but not laterally, by the direct transformation of shoot meristem. Alternatively in rice, the ovule is differentiated laterally from the FM. Furthermore, in *Arabidopsis*, the ovules originate from the margin of the carpel, which is differentiated laterally from the FM. If our presumption about sporangium

differentiation in the earliest vascular plants is true, it suggests that the pattern of ovule differentiation has changed from apical to lateral during the evolution of vascular plants. One convincing advantage of the lateral ovule differentiation pattern is that one shoot meristem can produce plural ovules, like in *Arabidopsis*. In this interpretation, the mode of rice, in which a single meristem produces a single ovule laterally, may be an intermediate pattern of ovule differentiation in vascular plants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.12.006.

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