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Neofunctionalization of B-class genes in regulating rice flower development

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A typical flower comprises four whorls of organs: sepal, petal, stamen, and pistil. Based on the floral quartet model (FQM), the identities of these floral organs are determined by various combinations of the ABCDE-class MADS-box proteins that function in overlapping domains within the flower[1]. The proliferation of the ABCDE genes in modern flowering plants is strongly correlated with the evolution and diversification of floral patterns, as exemplified by the independent duplication of B-class genes that led to petal derivation in the Orchid and Liliaceae families[2].

Spikelet, the unique flower structure of grass inflorescence, contains highly specialized non-reproductive organs[3]. In rice for example, each spikelet comprises two pairs of bract-like organs and a floret that consists of lemma and palea in the first whorl, two lodicules that are generally regarded as petal equivalent in the second whorl[4], six stamens in the third whorl, and one pistil in the center whorl (Fig. 1a). *OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS20* are A-class genes that regulate palea development. *OsMADS2*, *OsMADS4*, and *OsMADS16* are B-class genes that act together with the A-class genes to determine lodicule development. *OsMADS3* and *OsMADS58* are C-class genes that act in combination with the B-class genes to control stamen formation. *OsMADS13* and *OsMADS21* are D-class genes that function together with the C-class genes to regulate ovule formation and development. *OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *OsMADS34* are rice SEP-like (E-class) genes, serving as the 'glume' genes that participate in all floral organ development processes by interacting with proteins from the other classes to specify floral organ identity and determinacy[5]. Additionally, OsMADS6 and OsMADS17 are AGAMOUS-LIKE6 (AGL6) homologs with similar function to the E-class proteins, whereby OsMADS6 is known to control floral meristem (FM) and floral determinacies[6]. OsMADS32, an orphan protein in monocotyledonous plants, regulates floral context by interacting with other floral homeotic proteins[7]. However, to what extent the FQM can be applied to the development of the non-reproductive organs in the spikelet is still largely unclear.

Plant genomes contain two main lineages of B-class genes, *PI/GLO* and *paleoAP3/DEF*, which arose before the emergence of angiosperms. In rice, OsMADS2 and OsMADS4 are paralogs in the PI/GLO family that play the same role as the paleoAP3/DEF ortholog, OsMADS16 (also named as SUPERWOMAN1), in specifying lodicule and stamen[8]. However, previous studies of *OsMADS2* and *OsMADS4* RNAi plants indicated that these two genes play unequal roles in lodicule morphogenesis[9]. Whether they function differentially in floral meristem (FM) activity and floral organ development remains elusive.

To further distinguish the function between *OsMADS2* and *OsMADS4*, the CRISPR-Cas9 system was used to generate targeted mutations within the two genes to obtain strong mutant alleles. Two mutational events were identified for *OsMADS2* : *Osmads2-1* has an 'A' insertion and *Osmads2-2* has a 'TT' insertion (Supplemental Fig. S1a), both causing a frameshift and premature translational termination of the protein (Supplemental Fig. S1b). Compared with the wild-type (WT) plant, there was no visible abnormal phenotype from the vegetative to the reproductive stage, except that the lodicules were extended during floret development in both alleles (Supplemental Fig. S2), which is consistent with a previous report[9]. For *OsMADS4*, two types of mutants were obtained: *Osmads4-1* has a deletion of 'T' and *Osmads4-2* has an insertion of 'T' (Supplemental Fig. S3a), both leading to a frameshift and premature translational termination of the protein as well (Supplemental Fig. S3b). Compared with the WT plant, both *Osmads4-1* and *Osmads4-2* displayed normal vegetative and reproductive growth and the floret contained normal lodicules and stamens (Supplemental Fig. S5), which is also consistent with the previously reported phenotypes of the *OsMADS4* RNAi plant[9].

Double mutants were then generated by crossing *Osmads2-1* with *Osmads4-1*. Abnormal spikelet phenotypes were observed in the double mutant that mimicked *Osmads16/spw1-1*[5], in which the lodicules were transformed into margin region of palea (mrp)-like organs and stamens into carpel-like organs (Supplemental Fig. S6). Interestingly, enlarged ovaries appeared in the inner whorl of *Osmads2-1 Osmads4-1* (Supplemental Fig. S6c). Therefore, the genetic analysis supports results from the previous study that *OsMADS2* and *OsMADS4* are functionally redundant with an essential role in determining the identities of lodicules and stamens, while *OsMADS2* also has a distinct role in lodicule morphogenesis[9]. RT-qPCR analysis of the expression of *OsMADS2*, *OsMADS4* and *OsMADS16* in the mutant lines revealed that, although *OsMADS2* and *OsMADS4* did not seem to impact each other's expression, the expression of *OsMADS16* increased significantly in the *Osmads4-1* mutant (Fig. 1b; Supplemental Fig. S7), suggesting that *OsMADS16* might compensate for the loss of *OsMADS4* through transcriptional upregulation.

A previous study showed that *OsMADS6* (*AGL6*-like gene) and *OsMADS3* (C-class gene) are also involved in determining floral organ identities and meristem fate[10]. To determine whether these genes interact genetically with *OsMADS2* and *OsMADS4*, double and triple mutants between *Osmads6-1*, *Osmads3-4* and *Osmads2-1* and *Osmads4-1* were generated. The spikelet of the *Osmads2-1 Osmads6-1* double mutant displayed defects in the outer three whorls, and ectopic glume-like organs and lodicule-stamen mosaic structures were present in whorls 2 and 3 (Supplemental Fig. S8a), which mimicked phenotypes of *Osmads6-1*[10]. Similarly, the *Osmads4-1 Osmads6-1* double mutant also contained abnormal spikelet structure, with glume-like structures enclosing the stamen filament, as well as a reduced number of stamens (Supplemental Fig. S8b). Furthermore, *Osmads2-1 Osmads4-1 Osmads6-1* triple mutants were made by crossing *Osmads2-1* with the *Osmads4-1 Omads6-1*(+/–) heterozygous double mutant. Homeotic transformation of lodicules and stamens into glume-like organs and abnormal pistils was observed in the triple mutant (Supplemental Fig. S8c), mimicking phenotypes of the *spw1-1 Osmads6-1* double mutant except that it lacked the secondary inflorescence inside the spikelet[10]. Taken together, the genetic evidence indicated

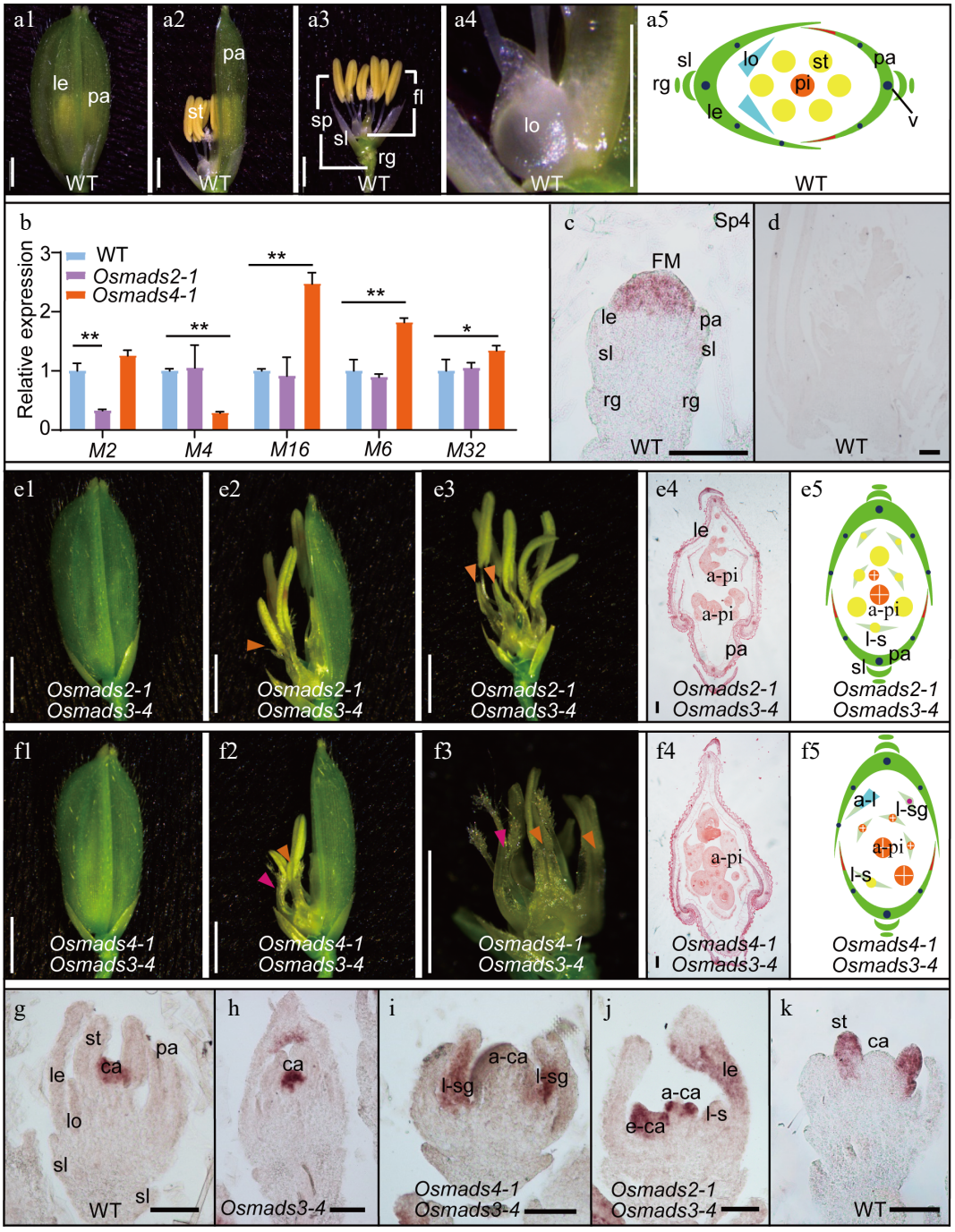

Fig. 1 (to be continued)

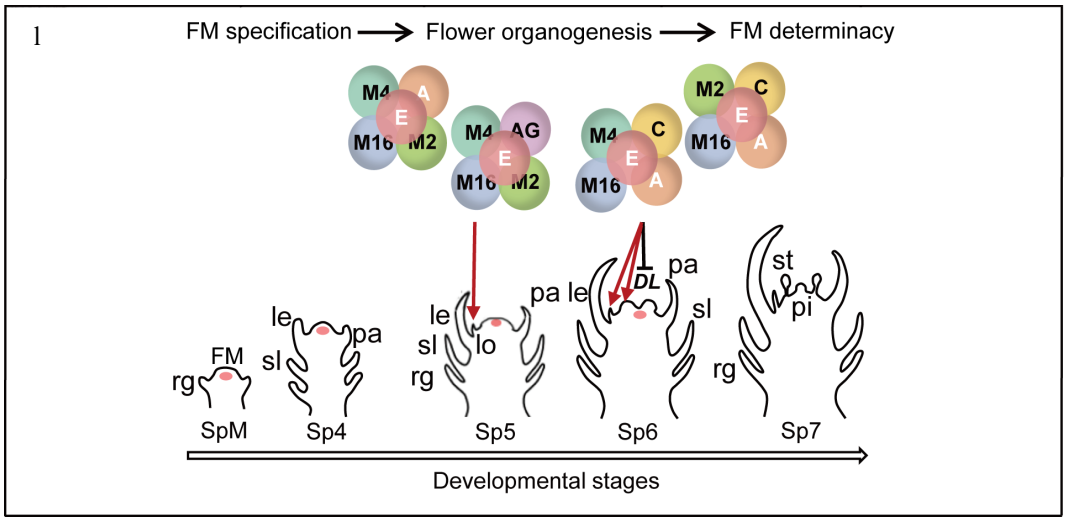

Fig. 1 OsMADS2 and OsMADS4 play partially distinct roles in lodicule and stamen specification. (a) Spikelet morphology (a1–a4) and cartoon diagram (a5) of Wild Type (WT). (b) Expression level of the B-class genes (*OsMADS2*, *OsMADS4* and *OsMADS16*), *OsMADS6* and *OsMADS32* in the 2-mm inflorescence of WT, *Osmads2-1* and *Osmads4-1*. Results are shown as mean ± SD. Error bars indicate SD for three biological replicates. ** indicates *p*-values < 0.01, * indicates P-values between 0.05 to 0.01, analyzed by Student-t test. (c), (d) *In-situ* hybridization analysis of *OsMADS4*. In the WT, signals for the anti-sense probe were detected in FM (c), whereas no signals for the sense probe were detected (d). Spikelet morphology (1–3), transverse section (4) and cartoon diagram (5) of the (e) *Osmads2-1 Osmads3-4* and (f) *Osmads4-1 Osmads3-4* double mutants. (g)–(j) *In-situ* hybridization analysis of *DL* in WT, *Osmads3-4*, *Osmads4-1 Osmads3-4* and *Osmads2-1 Osmads3-4*, respectively. (k) *In-situ* hybridization analysis of *OsMADS4* in WT. (l) A working model of the function of OsMADS2 and OsMADS4 in regulating rice flower development. OsMADS2 and OsMADS4 may engage with different protein complexes in specifying lodicule and stamen identity and morphogenesis. To specify lodicule identity, OsMADS2 and OsMADS4 play partially redundant roles in forming a complex with OsMADS16, the A-class proteins, AGL6-like proteins, and/or E-class proteins. OsMADS2 plays an additional role in regulating lodicule morphogenesis. On the other hand, OsMADS4 may form a protein complex with OsMADS16, and C-, A- and E-class proteins to determine stamen identity. OsMADS4 has a specific role in inhibiting the expression of *DL* in the lodicule and stamen to specify their identity and morphogenesis. Orange arrowheads in (e) and (f) indicate lodicule-stamen mosaic organs; Rose arrowheads in (e) indicate lodicule-stigma mosaic organ. a-ca, abnormal carpel; a-pi, abnormal pistil; e-ca, ectopic carpel; fl, floret; FM, floral meristem; le, lemma; lo, lodicule; l-s, lodicule-stamen mosaic organ; l-sg, lodicule-stigma mosaic organ; pa, palea; pi, pistil; rg, rudimentary glume; sl, sterile lemma; sp, spikelet; st, stamen. Scale bars = 2 mm in a1 to a4, e1 to e3 and f1 to f3; scale bars = 100 μm in e4 and f4; and scale bars = 50 μm in g to k. Red arrows in l indicate positive regulation in flower organ identity specification. The black bar in l indicates negative regulation. A, A-class proteins; AG, AGL6-like proteins; C, C-class protein; E, E-class proteins; SpM, spikelet meristem. M is the abbreviation of OsMADS; *DL*, *DROOPING LEAF*. Sp refers to a developmental stage of rice spikelet.

that *OsMADS2* and *OsMADS4* are partially redundant with *OsMADS6* in specifying lodicule and stamen identities. Unlike OsMADS16/SPW1, OsMADS2 and OsMADS4 may not participate in regulating secondary inflorescence growth and FM determination.

The C-class gene mutant *Osmads3-4* displayed mild homeotic transformation of lodicules and stamens into lodicules-like or lodicule-anther mosaic organs in the spikelet (Supplemental Fig. S9a–c)[11]. In *Osmads2-1 Osmads3-4,* some lodicules were transformed into lodicule-stamen organs (Fig. 1e; Supplemental Fig. S9d–f), whereas the ectopic expression of the stigma identity gene, *DROOPING LEAF* (*DL*), was observed in the ectopic carpels (Fig. 1j). On the other hand, the *Osmads4-1 Osmads3-4* double mutant displayed new phenotypes, including the transformation of lodicules into lodicule-stigma or lodicule-stamen mosaic organs (Fig. 1f; Supplemental Fig. S9g, h), and ectopic generation of abnormal carpels in whorl 3 (Fig. 1f; Supplemental Fig. S9g, i). These data suggest that in the absence of OsMADS3 and OsMADS4, OsMADS58 alone cannot fully determine the stamen identity. It is possible that in the *Osmads3 Osmads4* double mutant, *OsMADS58* together with *DL* are ectopically expressed in the second whorl to help specify stamen identity.

RT-qPCR analysis showed that the expression of *DL* increased significantly in the *Osmads4-1* single mutant at the floral maturation stage (Supplemental Fig. S10a), which prompted *in-situ* hybridization to investigate the expression pattern of *DL* in the mutants. Compared to WT and *Osmads3-4* (Fig. 1g, h; Supplemental Fig. S4), ectopic expression of *DL* was detected in the lodicule-stigma mosaic organs of *Osmads4-1 Osmads3-4* (Fig. 1i). The combined data suggests that, while the two rice PI-like proteins, OsMADS2 and OsMADS4, play redundant roles in lodicule and stamen specification, they might form different protein complexes in specifying lodicule and stamen identities. Besides its role in lodicule morphogenesis, OsMADS2 also genetically interacts with OsMADS3 in specifying stamen identity. OsMADS58 may need to form a complex with OsMADS4 to better specify stamen identity, and when only OsMADS2 is available, the formation of OsMADS58-SEP tetramers are favored to determine the carpel identity.

Since the expression of *OsMADS16* and *DL* increased in the *Osmads4-1* mutant, we performed RT-qPCR to detect transcript levels for all the MADS-box genes known to be involved in the development of lodicule and other reproductive organs to further understand the role of OsMADS4. Interestingly, expression of the E-class genes *OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8* and *OsMADS34* (Supplemental Fig. S10b–f), as well as *OsMADS32* and *OsMADS6,* was obviously increased during early spikelet development in *Osmads4-1*, but not in *Osamds2-1* (Fig.

1b; Supplemental Fig. S10g, h). A similar expression pattern was observed for *AP1*-like genes *OsMADS14* and *OsMADS15* and *OsMADS58* (Supplemental Fig. S10i–k), suggesting that OsMADS4 may have evolved a function in regulating rice floral meristem development. Previous evidence indicated that changes in gene expression and/or protein function might cause functional divergence for duplicated genes[1]. The *in-situ* hybridization analysis detected *OsMADS4* transcripts in the FM at Sp4 and stamen at Sp7, similar to those of *OsMADS2*[12], but hardly detected signals in the FM at Sp7 (Fig. 1c, k), which is different from a previous report in which the expression of *OsMADS4* was detected in carpel primordium[12]. This discrepancy might have been caused by the stage of the analyzed materials, as the floret used in the present study was at early Sp7, whereas the previous report used material at the late Sp7 stage. Together, these results indicate that the spatial-temporal expression of the rice PI-like genes as well as the formation of their protein complexes are key mechanisms that drive their specific functions.

In Arabidopsis, the B-class genes regulate FM maintenance and termination in an AG-dependent manner, as the AG/SEP-AG/SEP complex can switch to the AG/SEP-AP3/PI quartets under the ectopic expression of AP3/PI proteins[13]. Extra mrp-like glumes and lodicule-stigma mosaic organs grew in the *Osmads4-1 Osmads3-4* (Fig. 1f), *spw1-1 Osmads3-4* and *spw1-1 Osmads58* double mutants[5], suggesting that rice B genes also play a conserved role in maintaining the size of FM. Therefore, the B-class proteins may be key regulators that determine stage-specific protein quartet complex formation during flower development, both at the transcription and protein levels. Exploring the specificities of different protein quartets at various stages of flower development would help us discern the spatial-temporal regulatory networks in FM maintenance and termination.

Similar to the present observation with *Osmads3 spw1-1*, a previous study also observed lodicule-stigma mosaic organs in the *spw1-1 Osmads58* double mutants[8]. It is therefore speculated that OsMADS4, OsMADS16, OsMADS3, and OsMADS58, together with E-class and AGL6-like proteins, might form different complexes in specifying lodicule, stamen, and pistil identities (Fig. 1l). Additional *in vivo* protein interaction data are needed in the future to support the concept of a 'complex transition' that occurs in different whorls. Our observation, along with the ectopic expression of the leaf- and stigma-specific gene *DL*, in the lodicule of the *Osmads4-1 Osmads3-4* mutants, suggests that rice lodicule is a bracteopetal organ derived from a modified leaf, rather than an andropetal stamen-derived organ. In this case, duplication of rice B-class genes may have contributed to the diversification of petal-like organs in grasses, just like in eudicot. To test this hypothesis, it will be important to elucidate in the future whether and how the OsMADS4-OsMADS3 complex represses the expression of *DL*.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yuan Z, Wang L; project supervision: Yuan Z; *Osmads2* and *Osmads4* CRISPR lines generation: Li QL; analysis and interpretation of results (experiments): Yuan Z, Wang L; draft manuscript preparation and revision: Wang L, Yuan Z, Hu JP. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data used in this work are publicly available.

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Conflict of interest

The authors declare that they have no conflict of interest.

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