The maternally expressed polycomb group gene OsEMF2a is essential for endosperm cellularization and imprinting in rice

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ABSTRACT

Cellularization is a key event in endosperm development. Polycomb group (PcG) genes, such as Fertilization-Independent Seed 2 (FIS2), are vital for the syncytium-to-cellularization transition in Arabidopsis plants. In this study, we found that OsEMF2a, a rice homolog of the Arabidopsis PcG gene Embryonic Flower2 (EMF2), plays a role similar to that of FIS2 in regard to seed development, although there is limited sequence similarity between the genes. Delayed cellularization was observed in osemf2a, associated with an unusual activation of type I MADS-box genes. The cell cycle was persistently activated in osemf2a caryopses, which was likely caused by cytokinin overproduction. However, the overaccumulation of auxin was not found to be associated with the delayed cellularization. As OsEMF2a is a maternally expressed gene in the endosperm, a paternally inherited functional allele was unable to recover the maternal defects of OsEMF2a. Many imprinted rice genes were deregulated in the defective hybrid seeds of osemf2a (♂)/9311 (♀) (m9). The paternal expression bias of some paternally expressed genes was disrupted in m9 due to either the activation of maternal alleles or the repression of paternal alleles. These findings suggest that OsEMF2a-PRC2-mediated H3K27me3 is necessary for endosperm cellularization and genomic imprinting in rice.

Keywords: endosperm, cellularization, osemf2a, PRC2, genomic imprinting, rice

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INTRODUCTION

A triploid endosperm is a product of double fertilization. For most higher plants, the primary endosperm cell divides into a syncytium that contains multiple free nuclei through cell cycles uncoupling with cytokinesis. Subsequently, the syncytium initiates cellularization, which is a key event in endosperm development (Olsen, 2004; Wu et al., 2016). Both precocious and delayed cellularization can lead to seed failure (Walia et al., 2009; Ishikawa et al., 2011; Sekine et al., 2013).

In Drosophila, polycomb repressive complex 2 (PRC2) is composed of four core polycomb group (PcG) proteins: WD40 protein p55, suppressor of zeste 12 (Su(z)12), enhancer of zeste, and extra sex combs (Tonosaki and Kinoshita, 2015). Although PRC2 is conserved in eukaryotes, plants have multiplied the group members of each PcG family during evolution (Furihata et al., 2016; Huang et al., 2017; Qiu et al., 2017). Due to their methyltransferase activity for the methylation of Lys27 in histone H3 (H3K27), PRC2 complexes are vital for plant development, as they modulate the epigenetic modification of chromatin (Mozgova and Hennig, 2015). For example, Fertilization-Independent Seed (FIS)-PRC2 is critical for endosperm development. Fertilization-Independent Seed 2 (FIS2), Fertilization-Independent Endosperm (FIE), MEDEA (MEA), and Multicopy Suppressor of IRA1 (MSI1), which constitute the core FIS–PRC2 in Arabidopsis plants, are key regulators in the repression of central cell proliferation in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997; Kiyosue et al., 1999;
After fertilization, many type I MADS-box genes are transiently activated during cellularization (Bemer et al., 2010; Zhang et al., 2018b), possibly antagonizing FIS–PRC2 (Pires, 2014). Aberrant activation of type I MADSs has been found to be associated with cellularization failure in Arabidopsis and rice (Walia et al., 2009; Ishikawa et al., 2011; Zhang et al., 2018b; Paul et al., 2020). For instance, agamous-like 62 (agl62) and agl80 mutants exhibit precocious cellularization (Portereiko et al., 2006; Kang et al., 2008), and the seed failures of the mea mutant and interspecific hybrids that are caused by delayed cellularization can be alleviated by the inactivation of PHERE1 (PHET), AGL62, and AGL90 in Arabidopsis (Köhler et al., 2003a; Walia et al., 2009). The expression of many type I MADS-box genes is regulated by FIS–PRC2 in Arabidopsis plants (Zhang et al., 2018b).

Genomic imprinting is a non-Mendelian phenomenon that predominantly occurs in the endosperm of plants (Gehring, 2013). For imprinted genes, due to epigenetic differences, only one of the parental alleles is expressed, whereas the other is largely, if not completely, silenced (Köhler et al., 2012). The deregulation of imprinted genes has been found to associate with the developmental defects of interplody and interspecific hybrid seeds (Julien and Berger, 2010; Kradolfer et al., 2013; Burkart-Waco et al., 2015; Florez-Rueda et al., 2016; Tonosaki et al., 2018). Several PcG genes, such as FIS2, FIE, and MEA, are imprinted (Kinoshita et al., 1999; Luo et al., 2000) and also involved in the regulation of genomic imprinting (Gehring, 2013).

To date, our knowledge on PRC2-regulated seed development is limited in species other than Arabidopsis. Similar to FIE in Arabidopsis, the rice homolog OsFIE1 is imprinted (Zhang et al., 2012). Although OsFIE1 plays a limited role in cellularization (Huang et al., 2016), it is likely involved in the regulation of seed dormancy (Cheng et al., 2020). The other FIE homolog in rice is OsFIE2 (Luo et al., 2009). Nallamilli et al. (2013) have reported that OsFIE2 knockdown causes filling defects in seeds. Moreover, we have recently found that OsFIE1 and OsFIE2 functionally diverged during evolution (Cheng et al., 2020). OsFIE2 is essential for early endosperm development, as evidenced by impaired cellularization in the osfie2 mutant. The maize genome also encodes two FIE homologs that exhibit distinct expression and imprinting patterns (Danilevskaya and Hermon, 2003). However, their functions in seed development remain elusive.

The members of each PcG group in different plant species are not conserved. For example, the Arabidopsis genome encodes three Su(z)12-like genes, namely, Embryonic Flower 2 (EMF2), Vernalization 2 (VRN2), and FIS2 (Xiao and Wagner, 2015), whereas the rice genome encodes only two, namely, OsEMF2a and OsEMF2b (Luo et al., 2009). Additionally, FIS2 is an essential component of the FIS–PRC2 complex for the repression of the central cell before fertilization and for the promotion of cellularization during endosperm development (Chaudhury et al., 1997; Haudhury, 1999; Luo et al., 2000). As FIS2 does not exist in plants other than Brassicaceae (Qiu et al., 2017), it is unclear whether Su(z)12 is involved in the regulation of seed development in non-Brassicaceae species. OsEMF2a and OsEMF2b are phylogenetically close to EMF2 in Arabidopsis (Luo et al., 2009). A previous study reported that OsEMF2b knockdown could reduce seed weight, but no obvious developmental defects were observed (Chen et al., 2017). In the present study, we show that OsEMF2a may play a similar role to FIS2 in endosperm development in rice.

RESULTS

Expression of OsEMF2a and OsEMF2b

In rice, OsEMF2a and OsEMF2b are close homologs of EMF2 (Luo et al., 2009). Our phylogenetic analysis indicated that all the diploid grass species we analyzed, except for maize, had EMF2a and EMF2b orthologs in their genomes (Supplemental Figure 1A). The maize genome encoded two EMF2b paralogs but not EMF2a (Supplemental Figure 1A). These results suggest that EMF2a and EMF2b evolved from an ancient duplication event that occurred before the origin of the Poaceae family.

Quantitative real-time PCR (qRT–PCR) analysis suggested that OsEMF2a and OsEMF2b were constitutively expressed in various tissues, including the shoot, root, leaf, leaf blade, panicle, pollen, and caryopsis of rice, with the highest abundance in the pollen (Supplemental Figure 1B). As the Su(z)12 family member FIS2 is indispensable for seed development, we next investigated the expression profiles of OsEMF2a and OsEMF2b in developing caryopses. The results revealed that OsEMF2a was more abundant than OsEMF2b after fertilization (Supplemental Figure 1C), suggesting a potential role of OsEMF2a in seed development in rice.

Phenotypic characterization of OsEMF2a mutants

The OsEMF2a mutants were generated in the Zhonghua11 (ZH11, Oryza sativa ssp. japonica) background via a CRISPR/Cas9-mediated approach. Using distinct guide RNAs for targeting, two independent homozygous mutants, osmemf2a-1 and osmemf2a-2, were obtained at the T0 generation. A single-base insertion was identified in both lines (Figure 1A and Supplemental Figure 2A), which resulted in truncated proteins due to frame shifts. The vegetative growth of osmemf2a-1 and osmemf2a-2 was not significantly affected, but the heading date was somewhat delayed in the mutants (Figure 1B). Notably, we did not observe floral defects or sterility in osmemf2a (Figure 1C) as in osmemf2b (Deng et al., 2017).

The osmemf2a seeds were morphologically similar to the wild-type (WT) seeds before desiccation (Figure 1D). However, the caryopses of osmemf2a were more transparent because they were filled with jelly-like, rather than milky or starchy, endosperm compared with that in the WT (Figure 1E–1G). The osmemf2a mature seeds were inviable because there was no accumulation of storage compounds after drying, which left empty caryopses in the seeds (Figure 1H). The mutants we used were propagated asexually by ratooning. As the phenotypes of different mutant lines were similar (Figure 1B and
Figure 1. Phenotype of the *osemf2a* mutants.

(A) Generation of the *osemf2a* mutants in the Zhonghua11 (ZH11) background via a CRISPR/Cas9 approach. T1 and T2 indicate the targets used for gene mutation. Both mutant lines had a single-base insertion to cause premature stop codons.

(B) Morphology of the wild-type (WT) (left), *osemf2a-1* (middle), and *osemf2a-2* (right) plants at the heading stage.

(C) Morphology of the WT (left) and *osemf2a-1* (right) flowers. The inset panels show the pollen activities of WT (left) and *osemf2a-1* (right) after staining with I2-IK solution.

(D) Morphology of the seeds produced by the WT (upper) and *osemf2a-1* (lower) at 10 days after fertilization (DAF).

(E and F) Morphology of the WT (E) and *osemf2a-1* caryopses (F) at 10 DAF.

(G) Bare-handed sections that show the starchy endosperm of the WT (upper) and the jelly-like endosperm of *OsEMF2a-1* (lower).

(H) Morphology of the caryopsis produced by the WT (upper) and *osemf2a-1* (lower) at the mature stage.

(I) Setting rate of the WT and *osemf2a-1*.

(J and K) H3K27me3 modifications in the chromatin of the endosperm (J) and leaf (K) of the WT (the first lane of each figure) and *osemf2a-1* (the second lane of each figure).

(L) Cumulative percentages of the non-fertilized ovules and enlarged, autonomous seeds in the WT, *osemf2a-1*, and *osemf2a-2*.

(M) Morphology of autonomous seeds produced by *osemf2a-1* at the mature stage.

(N) Confocal laser scanning microscopy (CLSM) observation of an artificially fertilized *osemf2a-1* caryopsis at 7 days after pollination. The red arrowhead and the yellow arrow show the embryo and endosperm, respectively. Scale bar, 100 μm.

(O–R) CLSM observation of the autonomous endosperm of *osemf2a-1* (O and P) and *osemf2a-2* (Q and R) caryopses at 7 days after emasculation. Embryos were not observed in the autonomous seeds. (P) and (R) are magnified images of the boxed regions in (E) and (Q), respectively. Scale bars, 100 μm.
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Supplemental Figure 2B–2E, we used osemf2a-1 for most of the downstream experiments unless otherwise specified. Notably, the setting rate of osemf2a was not different from that of the WT (Figure 1I), suggesting that its fertilization was not affected.

As OsEMF2a is a PcG-encoding gene, we compared the trime-thylation at H3K27 (H3K27me3) in the WT and mutant chromatin. Not surprisingly, H3K27me3 was significantly reduced in the osemf2a caryopsis (Figure 1J), indicating that OsEMF2a-PRC2 has methyltransferase activity in planta. However, we did not detect decreased H3K27me3 in the leaf (Figure 1K), possibly due to the redundancy of OsEMF2b. In line with this, we did not observe vegetative defects in osemf2a.

Autonomous endosperm of OsEMF2a

The Arabidopsis PRC2 mutants can produce autonomous seeds (Chaudhury et al., 1997; Vinkenoog et al., 2000). To test whether the mutation of OsEMF2a results in autonomous seeds in rice, we emasculated the mutant spikelets on the day before flowering. A small proportion of the emasculated seeds (3.7% in osemf2a-1 and 3.1% in osemf2a-2) were able to develop further but failed to accumulate storage compounds, leaving enlarged, yet unfilled, caryopses in seeds at the mature stage (Figure 1L and 1M). We assumed that these enlarged seeds were autonomous. To confirm this hypothesis, we visualized the enlarged seeds produced by emasculated osemf2a via confocal laser scanning microscopy (CLSM). As a control, the artificially fertilized osemf2a seeds developed embryos in the embryo sacs at 7 days after fertilization (DAF) (Figure 1N), despite the development being abnormal (see below). By contrast, there was no embryo observed in the enlarged seeds that were produced by emasculated osemf2a at 7 days after emascula-tion (Figure 1O–1R). However, nuclear division of the central cell was observed in the enlarged seeds produced by emasculated osemf2a-1 and osemf2a-2 (Figure 1P and 1R), which indicated that the enlarged seeds were autonomous.

Delayed cellularization and arrested embryo development of sexually produced osemf2a seeds

Our histological analysis indicated that the syncytial endosperm of a WT seed initiated cellularization at 3 DAF and completed it at 4 DAF (Figure 2A–2D and Supplemental Figure 3A–3E). However, for the osemf2a mutant, the sexually produced endosperm cells were still syncytial at 5 DAF (Figure 2E–2H and Supplemental Figure 3F–3J). The delayed cellularization of osemf2a was confirmed via CLSM (Figure 2I and 2J). Cellularized endosperm cells of osemf2a were observed at 7 DAF but were disorderly aligned in the caryopsis (Supplemental Figures 3E and 3J).

Embryo development in osemf2a generally ceased at the globular stage (Figure 2K–2S). Few osemf2a embryos developed into a further stage at 10 DAF, and their development lagged behind that of the WT (Figure 2S). As no viable seeds were produced by osemf2a, we believe that the further-developed embryos were not vigorous due to developmental defects in the endosperm.

Accumulation of storage compounds was destroyed in the endosperm of osemf2a

The cellularized endosperm cells of osemf2a did not exhibit the I$_2$–KI staining reaction (Figure 3A–3D), indicating that the mutant failed to accumulate starch in its caryopsis. In agreement, transmission electron microscopy (TEM) analysis revealed that unlike in WT cells, no starch granules were observed in the sexually produced endosperm cells of osemf2a-1 (Figure 3E and 3F). After measuring the starch accumulated in the 10-DAF-old caryopses, we found that the content of the osemf2a-1 mutant was only 8% that of the WT (Figure 3G). Nevertheless, the mutant accumulated more sucrose, fructose, and glucose in its caryopsis (Figure 3H–3J), implying that the defective starch biosynthesis of osemf2a was not due to the lack of sugar supplements. In addition, the synthesis of storage proteins was nearly abolished in the mutant (Supplemental Figure 4). Therefore, we believe that the accumulation of storage compounds was substantially impaired in the seeds of osemf2a. Compared with the WT, the I$_2$–KI staining reaction was stronger in the pericarp of osemf2a caryopses (Figure 3C and 3D). In addition, unlike the WT, aleurone cells were not observed in osemf2a seeds at 7 DAF (Figure 3A–3D). These findings suggest that OsEMF2a may also be involved in the maternal development and endosperm differentiation of rice seeds.

Next, RNA sequencing (RNA-seq) was performed to unravel the transcriptional changes in the caryopsis at 10 DAF. Compared with the WT, 2992 genes were upregulated and 933 genes were downregulated (fold change >2, false discovery rate [FDR] <0.05) in osemf2a-1 (Supplemental Table 1). MapMan analysis of the differentially expressed genes (DEGs) suggested that several pathways, such as those for starch synthesis and storage proteins, were significantly enriched (Supplemental Table 2). For example, many genes involved in starch biosynthesis, namely, AGPS2, AGPL2, OsSSIIIa, and OsSSIIIa, were substantially repressed in the caryopsis of OsEMF2a (Figure 3K and Supplemental Figure 5).

Delayed cellularization of osemf2a associated with overactivated cell cycles

In agreement with the enlarged syncytium and delayed cellularization observed in the endosperm of osemf2a (Figure 2A–2H), the RNA-seq data revealed that genes involved in DNA synthesis, such as histone genes, were also overrepresented in the upregulated DEGs (Supplemental Table 2). Therefore, we suspected that the overproliferation of the syncytial endosperm cells of osemf2a was partially due to the overactivation of the cell cycles. In support of this hypothesis, numerous cell cycle-related genes were upregulated in osemf2a-1. For example, 16 of the 46 cyclin-encoding genes identified from the rice genome were differentially expressed, most of which (15/16) were upregulated (Figure 4A). The expression of two cell cycle-related genes, Orysa:CycB1;1 and Orysa:KRPI;1, which function in endosperm development (Barróco et al., 2006; Guo et al., 2010), was significantly increased in the caryopsis of osemf2a at the early endosperm development stage (Supplemental Figure 6).

Coincidentally, many genes involved in cytokinin metabolism and signaling, such as OsLOGL1, OsIPT2, OsRR5, and OsRR13, were significantly upregulated in osemf2a-1 (Figure 4B and Supplemental Table 1). The levels of four active cytokinin forms, namely, zeatin, trans-zeatin riboside (tZR), isopentenyladenine (iP), and isopentenyladenine riboside (iPR),
were significantly higher in the mutant caryopsis at 5 DAF (Figure 4 C–4F). Similar to the WT, zeatin and tZR levels significantly decreased in the mutant at 10 DAF, but their levels were still 113- and 57-times greater than those of the WT, respectively (Figure 4C and 4D). At 10 DAF, the accumulation of iP and iPR in the mutant was even higher than that at 5 DAF (Figure 4E and 4F).

Compared with the WT, less auxin was accumulated in the caryopsis of *osemf2a-1* at 5 and 10 DAF (Supplemental Figure 7A). Interestingly, we found that *OsYUC9* and *OsYUC11*, two active auxin biosynthetic genes in the WT, were completely silenced in the mutant (Supplemental Figure 7B and 7C). In the WT, *OsYUC12* was transiently activated at 3–5 DAF but was not activated until 7 DAF in *osemf2a* (Supplemental Figure 7D). Along with the induction of *OsYUC12, OsYUC13* and *OsYUC14* were activated simultaneously, whereas these genes were not expressed in the WT (Supplemental Figure 7E and 7F). These findings indicate that the cellularization defects in rice were not associated with the overproduction of auxin. Thus, we inferred that the overproliferated syncytium of *osemf2a* was caused by the activated cell cycles, which were likely triggered by the overproduced cytokinins in the caryopsis.

**Type I MADS-Box genes were overactivated in the caryopsis of *osemf2a***

As revealed by the RNA-seq data, the expression of many genes in the MADS-box family was disrupted (Supplemental Table 3 and Supplemental Figure 8). Given their importance in seed development, we focused on type I MADS-box genes for analysis. The majority of type I OsMADSs exhibited extremely low
Figure 3. Storage compound accumulation defects in osemf2a.
(A and B) Transverse sections of 7-DAF-old caryopses of the WT (A) and osemf2a-1 (B). The sections were stained with toluidine blue. Pe, Al, and En indicate pericarp, aleurone, and cellularized endosperm cells, respectively. Scale bars, 50 μm.
(C and D) I2-IK staining of 7-DAF-old caryopses of the WT (C) and osemf2a-1 (D). Pe, pericarp; En, cellularized endosperm cells. Scale bars, 50 μm.

[legend continued on next page]
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expression levels in the WT caryopsis at 10 DAF; however, many of them, including OsMADS70, 77, 79, 81, 82, 83, 87, 89, and 90, were significantly upregulated in osemf2a (Figure 5A). After analyzing the expression dynamics of OsMADS77, 79, 82, and 89, we found that these genes were persistently activated in the mutant at 1–2 DAF (Figure 5B–5F).

Our phylogenetic analysis indicated that OsMADS70, 77, and 79 have close relationships with AGL62, whereas OsMADS81, 82, 83, 87, and 89 are closer to Arabidopsis PHE1 and PHE2 (Figure 5G). Similar to PHE1, OsMADS87 is an imprinted gene, but it predominately expresses the maternal allele in rice (Ishikawa et al., 2011; Tonosaki et al., 2018). All of these genes were activated in the osemf2a caryopsis at 10 DAF (Figure 5A–5F). Previous studies have revealed that the suppression of AGL62 or PHE1 could recover the endosperm collapse in FIS–PRC2 mutants (Köhler et al., 2003a; Hehenberger et al., 2012).

As OsMADS77, 79, 87, and 89 were the top four activated type I MADS-box genes in the osemf2a caryopsis, we attempted to simultaneously knockout OsMADS77, 79, 87, 89, and OsEMF2a. Due to sequence similarities among OsMADS77, 79, and 79, we were able to use a single-guide RNA to knockout all three genes. We also designed a degenerate guide RNA in an attempt to target OsMADS87 and 89. Therefore, a CRISPR/Cas9 cassette that included three tandemly arrayed guide RNAs (one for OsEMF2a, one for OsMADS77/87/89, and one for OsMADS87) was able to target six genes. However, due to the sequence differences between OsMADS87 and OsMADS89, we failed to obtain OsMADS89 mutations among the 24 transformants we generated. Nevertheless, using this strategy, we were able to obtain a quintuple mutant of osemf2a/osmads77/osmads78/osmads79/osmads87 at the T0 generation. There were as follows: 1-bp deletion in OsMADS77; 1-bp insertion in OsMADS78; 1-bp insertion and 1-bp deletion in the two alleles of OsMADS79, respectively; 21-bp deletion in OsMADS77; and 1-bp and 3-bp deletion in the two alleles of OsEMF2a, respectively (Supplemental Figure 9A). However, all seeds produced by the quintuple mutant were abnormal and resembled the seeds produced by osemf2a (Supplemental Figure 9B and 9C), suggesting that disturbances in OsMADS77, 79, 87, and 89 are unable to (or insufficient to) recover the impaired endosperm development of osemf2a.

To answer whether the type I MADS-box DEGs are likely direct targets of the OsEMF2a–PRC2 complex, we analyzed the histone methylation status of OsMADS77 and OsMADS79 via the chromatin immunoprecipitation (ChIP) approach with an H3K27me3 antibody, followed by quantitative PCR (qPCR). For each gene, six qPCR primers (P1–P6) were designed for the promoter and gene body regions (Figure 5I and 5J). The ChIP–qPCR results showed that P2, P3, P4, and P6 of OsMADS77, and P1, P2, P4, and P6 of OsMADS79 were enriched for H3K27me3 in the WT caryopsis compared with that of the osemf2a-1 mutant at 10 DAF (Figure 5H and 5I). This finding supports the hypothesis that these overactivated type I MADS-box genes are downstream targets of the OsEMF2a–PRC2 complex in the endosperm.

OsEMF2a displayed a parent-of-origin-dependent expression pattern

We found that the hybrid seeds produced by the reciprocal cross of ZH11 and osemf2a displayed a distinct phenotype (Figure 6A and 6B). When osemf2a-1 was used as the pollen donor (Zm, ZH11 (♂/osemf2a-1 (♀)), >93% of the seeds it generated were well filled, with no empty seeds observed (Figure 6B). However, for the opposite direction of the cross (mZ, osemf2a-1 (♀)/ZH11 (♂)), ~66.9% and 26.5% of hybrid seeds were completely empty and poorly filled, respectively (Figure 6B). The rate of partially filled and well-filled seeds was higher than that of the osemf2a-1 (♀)/osemf2a-1 (♂) (mm) cross (Figure 6B). However, only one out of the 45 well-filled or partially filled seeds produced by m2 successfully developed into a seedling, the rest were unable to germinate on half-strength Murashige and Skoog medium. Similar results were obtained when osemf2a-2 was used for crossing (Supplemental Figure 10). A functional paternal allele that failed to recover the maternal defect in the hybrid led us to assume that OsEMF2a is a maternally expressed gene (MEG). To confirm this, we generated reciprocal crosses of ZH11 and 9311 (O. sativa ssp. indica). Using a single-nucleotide polymorphism (SNP) within the coding sequence (Figure 6D), we were able to distinguish the parental origin of the OsEMF2a transcripts. As expected, only the maternal allele was detected in the 10-DAF-old hybrid endosperm (Figure 6D). We also generated osemf2a-1 and 9311 reciprocal crosses. Once again, the functional OsEMF2a of 9311 failed to recover the dysfunctional maternal osemf2a (Figure 6C). Interestingly, in the osemf2a (♀)/9311 (♂) (m9) hybrids, we found that OsEMF2a became a biallele-expressed gene (Figure 6D), suggesting that OsEMF2a may regulate the imprinting of OsEMF2a either directly or indirectly.

OsEMF2a defect caused imprinting disorders in the endosperm

We next collected endosperm cells from 10-DAF-old caryopses produced by the reciprocal crosses of ZH11 and 9311 (Z9–9Z). An RNA-seq approach was used to identify genes that show a parent-of-origin-dependent expression pattern, taking advantage of the SNPs that can trace the parental origin of short RNA-seq reads. OsMADS29, a gene predominantly expressed in the maternal tissues of the rice caryopsis (Yin and Xue, 2012), was used as a marker to detect the maternal contamination. As revealed by qRT–PCR analysis, the expression of OsMADS29 was extremely low in the hybrid endosperm samples used for RNA-seq (Supplemental Figure 11), indicating that there was very limited maternal contamination, if any at all.

(E and F) Transmission electron microscopy analysis of 7-DAF-old endosperm cells of ZH11 (E) and osemf2a-1 (F). The yellow arrows indicate starch granules in the endosperm cells. Scale bars, 10 μm.

(G) Total starch content in the endosperm of the WT and osemf2a-1 at the mature stage. **p < 0.05, Student’s t-test for statistical analysis. The error bars indicate standard deviations; three biological replicates were used for the analysis.

(H–J) Sucrose (H), fructose (I), and glucose (J) contents of the WT and osemf2a-1 caryopses at 10 DAF. **p < 0.05, Student’s t-test. The error bars indicate standard deviations; three biological replicates were used for the analysis.

(K) Heatmap of the expression of key genes involved in starch biosynthesis. The expression level is indicated by the reads per kilobase per million mapped reads (RPKM) value. The color bar indicates the log2(RPKM).
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Using a threshold of more than 5-fold deviations from the 2m:1p expected (greater than 10m:1p for a MEG or less than 2m:5p for a paternally expressed gene [PEG]), we identified 114 strong PEGs and 26 strong MEGs with high confidence (FDR <0.05) from Z9–9Z (Supplemental Table 4). Over 78% (89/114) of the PEGs and 26 strong MEGs with high confidence (FDR <0.05) from Z9–9Z were common to previously identified imprinted genes in rice (Supplemental Table 5). The number of MEGs was much lower here than in previous studies using different genetic combinations, possibly due to the different age of the endosperm used for this investigation (10 DAF in the present study versus 5–7 DAF in previous studies). Due to the stringent cutoffs we set here, OsEMF2a was not identified as a strong MEG. The maternal allele consisted of 87% and 95% of the total OsEMF2a transcripts expressed in the ZH11 (♂)/9311 (♀) (Z9) and 9311 (♀)/ZH11 (♂) (9Z) endosperms, respectively (Supplemental Figure 12A). For the same reason, OsFIE1, a conserved MEG as revealed by previous studies, did not reach the threshold for a strong MEG (Supplemental Figure 12B). Therefore, the number of MEGs might have been underestimated here.

Next, we investigated the parent-of-origin-dependent expression pattern of these imprinted genes in m9, which exhibited seed developmental defects similar to those in osemf2a (Figure 6C). When we defined a gene whose maternal transcripts consisted of 50%–80% of the total transcripts (no more than a 2-fold deviation from the 2m:1p expected) as a biallelic-expressed gene, 47 strong PEGs and four strong MEGs of Z9–9Z exhibited disrupted imprinting in m9 (Figure 7A and 7B; Supplemental Table 5) and most showed biallelic expression in the m9 hybrids, implying that the imprinting of these genes is regulated by OsEMF2a. However, the majority of these genes did not change the paternal or maternal expression bias in 9311 (♀)/osemf2a (♂) (9m) (Figure 7A and 7B; Supplemental Figure 13). The results were consistent with those revealed by the RNA-seq data (Figure 7B and Supplemental Figure 4), implying that −41% (47/114) of the PEGs and 15% (4/26) of the MEGs of Z9–9Z exhibited disrupted imprinting in m9.

Activation of the maternal allele caused the imprinting disorders of many PEGs in m9

Overall, the expression of the imprinted genes in 9m was similar to that in 9Z (Figure 7C); however, the genes in 9m exhibited...
Figure 5. Overactivation of type I OsMADSs in osemf2a.

(A) Heatmap of the expression of type I MADS-box genes in 10-DAF-old caryopses of the WT and osemf2a-1. The color bar indicates the RPKM values of the genes in three biological replicates.

(B–F) Expression dynamics of OsMADS77 (B), OsMADS79 (C), OsMADS82 (D), OsMADS87 (E), and OsMADS89 (F) in WT and osemf2a-1 caryopses of different ages.

(G) Neighbor-joining tree of the type I MADS transcription factors of rice. PHE1, PHE2, and AGL62 in Arabidopsis are highlighted in red.

(H and I) ChIP–PCR assay showed that OsMADS77 (H) and OsMADS79 (I) were highly enriched for H3K27me3 in the endosperm of WT compared with that in the endosperm of osemf2a-1 at 10 DAF. Six investigated regions (P1–P6) are indicated in the schematic figures for each gene. Black boxes and gray bars indicate the coding sequences and the promoters of the genes, respectively. Error bars indicate standard deviations (n = 3). The ChIP–PCR assay was performed twice with consistent results; one of the experiments is presented.
substantial deregulation compared with those in Z9 (Figure 7D). These results suggest that the endosperm abortion of m9 is at least partially associated with the expression disorders of imprinted genes. Following this, we focused on the disrupted imprinted genes. Overall, the majority of these genes were activated in the defective m9 endosperm (Figure 7E and Supplemental Table 6). When a threshold fold change >2 and FDR <0.05 were used, 13 and eight dPEGs were significantly upregulated and downregulated, respectively (Supplemental Table 6). After analyzing the expression of the parental alleles, we found that the upregulated dPEGs were largely due to the activation of the maternal alleles in m9 (Figure 7F). For example, with regard to LOC_Os05g04330, which encodes Domains Rearranged Methyltransferase 3 in rice (OsDRM3), the maternal-origin transcripts (estimated by the read counts) of the gene in the m9 endosperm increased more than 10-fold compared with that in Z9; however, the abundance of the paternal-origin transcripts increased less than 1.5-fold (Figure 7F). Meanwhile, more severe suppression of the paternal allele was found in all eight downregulated dPEGs; for some genes, however, such as LOC_Os10g29549 and LOC_Os10g04980, both of the parental alleles were suppressed in m9 (Figure 7G). Three out of the four dMEGs exhibited differential expression and were significantly downregulated in the hybrid endosperm of m9 (Supplemental Table 6). For these genes, the maternal alleles exhibited a more severe suppression that disturbed the expression bias (Figure 7H).

We then developed derived cleaved amplified polymorphic sequence (dCAPS) markers for RT–PCR to verify the disproportional activation or repression of the parental alleles of each gene. After restriction digestion, we were able to detect the expression changes of the parental alleles in m9. The results were consistent with those revealed by the SNP counts (Supplemental Figure 14). For example, the dPEGs OsDRM3, LOC_Os03g03460, and LOC_Os11g34270 exhibited activation of the maternal alleles in m9 (Supplemental Figure 14), whereas LOC_Os11g68510 and LOC_Os10g04980 exhibited suppression of the paternal alleles (Supplemental Figure 14). For the dMEGs LOC_Os10g39420 and LOC_Os05g34310, the maternal alleles were significantly suppressed in m9 compared with those in Z9 (Supplemental Figure 14).

**DISCUSSION**

The two Su(z)12-group PRC2 members of rice are OsEMF2a and OsEMF2b (Luo et al., 2009), which likely evolved from an ancient duplication event before the origin of the family Poaceae (Supplemental Figure 1A). Previous studies reported that the osemf2b mutant displayed abnormal floral organs, reduced pollen fertility, and decreased dormancy (Xie et al., 2015; Chen et al., 2017; Deng et al., 2017; Zhong et al., 2018). However, we did not identify floral development and fertility issues in osemf2a (Figure 1C and 1I). Instead, we observed delayed cellularization of the endosperm cells and halted embryo development of osemf2a (Figure 2A–2S); these phenotypes were not observed in osemf2b. These findings suggest that despite their similar expression profiles (Supplemental Figure 1B and 1C), OsEMF2a and OsEMF2b are functionally divergent. Given that both osemf2a (Figure 1B) and osemf2b mutants (Xie et al., 2015) showed the late-flowering phenotype, we assumed that there are redundancies between the genes for heading. EMF2 in Arabidopsis functions in flowering regulation as well (Chanvivattana et al., 2004), indicating that its function in the vegetative-to-reproductive transition is conserved in plants. Nevertheless, it is worth noting that EMF2 defects promote flowering in Arabidopsis (Chanvivattana et al., 2004) but delay flowering in rice (Xie et al., 2015).

Previous studies have shown that FIS–PRC2 in Arabidopsis is important for endosperm development; fis2, fie, and mea mutants exhibit impaired cellularization and, thus, seed abortion (Kiyosue et al., 1999; Luo et al., 2000; Vinkenoog et al., 2000; Hennig, 2003). FIS2 was derived from a VRN2-like ancestor through whole-genome duplication (Qiu et al., 2017), whereas VRN2 was duplicated from EMF2 (Chen et al., 2009). Therefore, EMF2 is older than the other Su(z)12 members in Arabidopsis. In fact, EMF2 homologs are widespread among plant lineages; VRN2 homologs are not found in monocots, whereas FIS2 is Cruciferae-specific (Huang et al., 2017). Moreover, EMF–PRC2 regulates vegetative growth and flowering but is not involved in
Figure 7. Disrupted imprinting in osemf2a-1. 
(A and B) The proportions of the maternal transcripts in the total transcripts of each imprinted gene in the ZH11-9311 reciprocal crosses (Z9–9Z) (A) and their corresponding proportions in the osemf2a-9311 reciprocal crosses (m9–9m) (B). The black and red dots indicate the paternally expressed genes (PEGs) and maternally expressed genes (MEGs), respectively, identified from the Z9–9Z. The genes indicated in the figures were selected for validation of the disrupted imprinting in the hybrid endosperm of m9. 
(C and D) The imprinted genes identified from Z9–9Z showed a similar expression level in the hybrid endosperm of 9Z and 9m (C), but exhibited expression disorders in m9 when compared with Z9 (D).

(legend continued on next page)
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seed development in Arabidopsis (Chen et al., 1997; Charvivattana et al., 2004). In the present study, we found that OsEMF2a is necessary for the cellularization of rice (Figure 2A–2J). We hypothesized that EMF2a in monocots likely plays a similar role to FIS2 in endosperm development in Arabidopsis. It is worth noting that there is no EMF2a homolog in the maize genome (Supplemental Figure 1A). We assumed that EMF2b may replace EMF2a in maize. As FIS2 and EMF2a lack homologs in several species (Furihata et al., 2016), a recent study suggested that these genes likely undergo a lower selective constraint, reflecting their elevated non-synonymous substitution rate/synonymous substitution rate (dN/dS values). Alternatively, these higher dN/dS values could be caused by adaptive evolution, namely, the neo-functionalization of genes (Furihata et al., 2016).

Activation of type I MADSs usually accompanies cellularization failure in Arabidopsis (Zhang et al., 2018b). Type I MADS-box genes are believed to antagonize PRC2 for cellularization (Pires, 2014). Similar to that observed in Arabidopsis, overaccumulation of type I MADS-box genes was observed in osemf2a (Figure 5A–5F). Interspecific crosses between some rice species can cause precocious cellularization that is associated with the activation of type I MADSs in the aborted endosperm (Ishikawa et al., 2011; Tonosaki et al., 2018). These findings suggest that type I MADS-regulated cellularization is employed in both monocots and dicots. However, in this study, by knocking out the highly activated type I MADS in rice, we failed to rescue the endosperm failure of osemf2a (Supplemental Figure 9). This could have been caused by gene redundancies, as there were at least ten type I OsMADSs activated in the osemf2a caryopsis (Figure 5A–5F and Supplemental Table 3). The loss-of-function of OsEMF2a resulted in reduced H3K27me3 modifications of OsMADS77 and OsMADS79 (Figure 5H and 5I). This is in line with previous findings in Arabidopsis, which suggested that FIS–PRC2 can repress the expression of type I MADS-box genes during endosperm cellularization (Zhang et al., 2018b). Moreover, type I MADS-box genes can possibly recruit FIS–PRC2 in the central cells and maintain H3K27me3 during endosperm development (Wang et al., 2020). OsEMF2b can regulate the expression of MADS via H3K27me3 modifications as well. A group of OsMADSs that were activated in the osemf2b mutant (Xie et al., 2015) were also activated in the osmf2a mutant (Supplemental Figure 7 and Supplemental Table 3), further confirming that there are redundancies between OsEMF2a and OsEMF2b.

Cell cycle regulation is important for endosperm development (Dante et al., 2014). For instance, Cyclin B1 is essential for syncytium development in both Arabidopsis and rice plants (Guo et al., 2010, 2018). Moreover, OsYUC12 is a cyclin-dependent kinase inhibitor gene, is also involved in early endosperm development in rice (Barroco et al., 2006). We observed an enlarged syncytium (Figure 2H), which was associated with the activation of many genes related to the cell cycle, such as OsYuc12, CcycB1:1 and OsYucKRP1:1, in osemf2a (Figure 4A and Supplemental Figure 6). The cell cycle is usually regulated by the phytohormone cytokinin (Schaller et al., 2014); coincidentally, we observed the activation of cytokinin biosynthesis genes in the caryopsis of osemf2a (Figure 4B–4F). We assumed that the cytokinin-modulated cell cycle could be the target of OsEMF2a–PRC2 for syncytial development. Auxin was recently found to be an internal signal for early endosperm development in Arabidopsis (Figureiroedo et al., 2013), and overaccumulation of auxin in paternal-excess interploidy seeds can inhibit cellularization (Batista et al., 2019). Mutations in OsEMF2a also led to inhibited cellularization (Figure 2A–2J). However, we found that there was no auxin overproduction coupling with the cellularization defects of osemf2a (Supplemental Figure 7A), which suggested that the cellularization failure caused by PCR2 defects may be different from that caused by parental genome imbalance. Moreover, OsYUC9 and OsYUC11, two auxin biosynthetic genes that are highly expressed in the developing caryopses of rice (Abu-Zaitoon et al., 2012), were almost completely repressed in osemf2a (Supplemental Figure 7B and 7C). Interestingly, OsYUC12, along with its close homologs OsYUC13 and OsYUC14 (Zhang et al., 2018a), was substantially upregulated in the endosperm of osemf2a (Supplemental Figure 7D–7F). Although OsYUC12 is transiently expressed in the endosperm of rice in parallel with cellularization (Abu-Zaitoon et al., 2012), we recently found that OsYUC12 mutants do not exhibit cellularization defects (unpublished data). In WT plants, OsYUC13 and OsYUC14 were not expressed throughout the entire life cycle. The activation of OsYUC12, OsYUC13, and OsYUC14 in the defective endosperm of osemf2a implies that these genes may contribute to the early endosperm development of rice. However, further investigations are required to determine their biological significance.

FIS2 in Arabidopsis is a maternally expressed imprinted gene (Luo et al., 2000). Luo et al. (2009) found that neither OsEMF2a nor OsEMF2b is imprinted, however, we genetically confirmed that OsEMF2a is an MEG in rice (Figure 6A–6D). The development of the hybrid seeds was not unusual when osemf2a was used as the paternal parent but was abnormal when osemf2b was used as the maternal parent (Figure 6A and 6B). Imprinting shows little conservation among plant species (Waters et al., 2013; Hatorangan et al., 2016; Chen et al., 2018b). However, many FIS–PRC2 members of plants are imprinted. For example, FIS2 and MEA in Arabidopsis (Kinosita et al., 1999; Luo et al., 2000), ZmFIE1 and ZmFIE2 in maize (Danilevskaya and Hermon, 2003; Ni et al., 2019), and OsFIE1 and OsEMF2a in rice (Zhang et al., 2012; Chen et al., 2018b) have been identified as imprinted genes. The underlying cause of the bias is unclear. The significance of the imprinting of FIS–PRC2 members and their evolutionary advantages for endosperm development must be investigated in the future.
OsEMF2a regulates cellularization of rice

Upon analyzing the transcriptome changes, deregulation of the imprinted genes was found in the defective m9 endosperm (Figure 7A and 7B). This is consistent with previous findings that endosperm abortion usually accompanies expression disorders of the imprinted genes (Jullien and Berger, 2010; Kradoffer et al., 2013; Burkart-Waco et al., 2015; Florez-Rueda et al., 2016; Tonosaki et al., 2018). Approximately 41% of PEGs and 15% of MEGs of Z9–9Z exhibited disrupted imprinting in m9. This finding is in line with the assumption that PRC2-mediated H3K27me3 is important for the imprinting of PEGs (Zhang et al., 2014; Moreno-Romero et al., 2016). There were two primary ways to disturb the paternal expression bias in m9. First, for the upregulated dPEGs, the activation was mostly contributed by the maternal alleles, which eventually changed the paternal-preferential expression to biallelic expression of the genes (Figure 7F). For the downregulated dPEGs, the maternal allele was repressed, but with limited impact on the maternal alleles (Figure 7G). We believe that for the first case, the maternal alleles of these genes were repressed in the WT due to the H3K27me3 deposition in the central cell. The classical model of PEG regulation suggests that maternal demethylation in the central cells can facilitate H3K27me3 deposition and, thus, suppress the maternal expression (Hsieh et al., 2011; Batista and Köhler, 2020). Some PEGs are independent of maternal demethylation. For these genes, repression of the maternal alleles may require central-cell-specific transcription factors that guide FIS–PRC2 to the target regions (Batista and Köhler, 2020). In both of these scenarios, mutation of OsEMF2a can result in a genome-wide depletion of H3K27me3 in the central cell of rice and, thus, activate the maternal expression in m9. However, with regard to the downregulated dPEGs in m9, it is more likely that the changes in gene expression are an indirect effect caused by developmental defects in the endosperm.

METHODS

Plant materials and growth conditions

The rice plants were grown in Yangzhou, Jiangsu Province, and Linghui, Hainan Province, China, during the summer and winter seasons, respectively, with regular water and nutrient management. The spikelets were marked on the day of anthesis. Caryopses of different ages were collected by removing the glums for subsequent experiments. Samples that showed obvious developmental deviations were discarded. In addition, the WT and mutant samples used for side-by-side comparisons were labeled on the same day in case of environment-induced developmental deviations. As no viable osemf2a seeds were available, the mutants used were propagated asexually by ratooning.

For the reciprocal crossing, spikelets of the mother parent were emasculated the day before flowering. The panicles with emasculated spikelets were then bagged for pollination. The endosperm was collected at 10 DAF as previously described (Chen et al., 2018b). The harvested endosperm of each hybrid seed was divided into two parts, half of which was used to extract DNA to determine whether the hybrid endosperm was “true” using SNPs or repeat-sequence polymorphisms between the parents. The corresponding halves of the true hybrids were used for subsequent experiments.

Generation of CRISPR/Cas9 mutants

The CRISPR/Cas9 approach was used for mutant generation as previously described (Zhiguo et al., 2018). All targets used for mutagenesis are listed in Supplemental Table 7. For each transformant, a 200- to 300-bp segment that embraced the targeting site was amplified for Sanger sequencing at the T0 generation to screen the mutations.

Sectioning, staining, and microscopic observation

The caryopses were fixed and infiltrated in FAA solution (2% formalin, 5% acetic acid, and 45% ethanol [v/v]) and stored at 4 °C for use. The samples were dehydrated through a gradient ethanol series and infiltrated with xylene for embedding in resin. The samples were then sectioned at 2.5 μm by a microtome (Leica EM). The sections were subsequently stained with 0.1% toluidine blue or I2-KI solution (80 mg KI, 10 mg I2 per ml).

CLSM was performed as previously described (Taira et al., 2015). Images of the PI-stained embryos and endosperm cells were taken with an LSM710 (Zeiss) microscope with excitation/emission wavelengths of 559/619 nm.

Quantification of starch, glucose, sucrose, and fructose contents

A starch quantification kit (DF-2-Y, Comin), glucose quantification kit (PT-2-Y, Comin), sucrose quantification kit (ZHT-2-Y, Comin), and fructose quantification kit (GT-2-Y, Comin) were used for the analyses according to the manufacturer’s instructions. The starch, glucose, sucrose, and fructose contents were determined by spectrophotometry at 620, 505, 480, and 480 nm, respectively.

Extraction of seed total protein

The 10-DAF-old caryopses of ZH11 and osemf2a were ground into powder using liquid nitrogen. The powder was transferred into 1.5-ml centrifuge tubes, and a 15 μl/mg leaching solution (125 mM Tris–HCl [pH 6.8], 4 M urea, 4% SDS, 5% 2-mercaptoethanol) was added to the tubes. After incubation at 37 °C for 3 h with shaking, the samples were centrifuged (15 000 rpm) at 4 °C for 20 min. Subsequently, supernatants were collected for SDS–PAGE.

Nuclear protein extraction and western blotting

Nuclear protein extraction was performed as previously reported (Cheng et al., 2020), and the extracted proteins were used for western blotting. Polyclonal antibodies for H3 (Abcam, cat no. ab1791) and H3K27me3 (Millipore, cat no. 07-449) were used. Three independent experiments were conducted with at least two biological replicates for each.

RNA extraction and real-time PCR assay

Total RNA was extracted from different rice tissues using a Plant RNA Kit (Omega). First-strand complementary DNA (cDNA) was synthesized using HiScript Q RT SuperMix for qPCR (Vazyme); the oligo–dT primer was used for the reactions. Quantitative real-time PCR was performed in the CFX Connect Real-Time PCR Detection platform (Bio-Rad) using the AceQ qPCR SYBR Green Master Mix (Vazyme). The rice proteasome gene (LOC_Os03g65430) was used as endogenous control. The primers used for the qRT–PCR are listed in Supplemental Table 7.
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RNA-seq, differential expression analysis, and identification of imprinted genes

The RNA isolated from 10-DAF-old caryopses of the WT and osemf2a-1 was used for RNA-seq. Three biological replicates for each sample were set; each replicate consisted of about 20 caryopses. The samples were submitted to Novogene (Tianjin) for library preparation and sequencing. For the DEG analysis, we used the CLC Genomics Workbench 12.0 software as previously described (Cheng et al., 2020). The DEGs were submitted to MapMAN for pathway analysis (Usadel et al., 2009). TTools was used for heatmap generation (Chen et al., 2018a).

The true hybrid endosperm (10 DAF) of the reciprocal crosses between ZH11 and 9311 and osemf2a-1 and 9311 was collected for RNA isolation. The RNA samples were submitted to BGI for RNA-seq using the MGISEQ-2000 platform. SNPs called from the RNA-seq data of ZH11 and 9311 endosperm samples were used to determine the parental origin of the transcripts. The approach used for the identification of imprinted genes was identical to that previously described (Chen et al., 2018b). Two biological replicates of the ZH11 and 9311 reciprocal crosses were used (each consisting of about 15 individuals), but no replicate was set for the osemf2a-1 and 9311 reciprocal crosses due to a lack of sample availability. The hybrid endosperm of osemf2a-2/9311 was also used for the analysis. To identify the DEGs in m9, we routinely analyzed the RNA-seq data of Z9 and m9 using CLC Genomics Workbench 12.0 with default parameters; osemf2a-1/9311 and osemf2a-2/9311 were treated as two replicates of m9.

Parental expression analysis of the disrupted imprinted genes

The RT–PCR products were amplified from the cDNA of ZH11 and 9311, and osemf2a-1 and 9311 reciprocal crosses. The primers used for the reactions contained derived digestion sites of restriction enzymes, and dCAPS was used to distinguish the parental origin of the transcripts. The dCAPS primers were designed by the online tool dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). The primer sequence and its corresponding enzymes are listed in Supplemental Table 7.

Chromatin immunoprecipitation analysis

ChiP assay was performed essentially as described previously (Li et al., 2018). Two grams of caryopses (10 DAF old) collected from the WT and osemf2a-1 were crosslinked in 1% formaldehyde for 10 min at room temperature and then quenched by glycerine (125 mMol/l). Chromatin was extracted and fragmented by sonication using the Bioruptor UCD-200 (Diagenode). Chromatin (20 μl) was saved at −20°C for input DNA, and 100 μl of chromatin was used for immunoprecipitation with the H3K27Me3 antibody (Cell Signaling Technology, #9733S). The precipitated and input DNAs were then used as the templates for qPCR reactions. Six primer sets for each gene were designed to detect H3K27me3 enrichment of the genes. The primers are listed in Supplemental Table 7.

Hormone extraction and quantification

Approximately 0.2 g of caryopsis was finely ground using a pestle in a precooled mortar that contained 5 ml of extraction buffer (isopropanol/hydrochloric acid). The procedure was performed as previously described (You et al., 2016). The purified product was then subjected to high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) using the Agilent 1290 platform. HPLC analysis was conducted using a ZORBAX SB-C18 (Agilent Technologies) column (2.1 × 150 mm; 3.5 mm). The solvents of mobile phases A and B consisted of methanol/0.1% methanoic acid and ultrapure water/0.1% methanoic acid, respectively. The injection volume was 2 μl. The MS/MS conditions were as follows: the spray voltage was 4500 V; the pressures of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively; and the atomizing temperature was 400°C.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

C.C. conceived the project; C.C., B.N., X.C., and M.P. designed the research; X.C., M.P., Y.Z., and B.N. performed the experiments; C.C., X.C., M.P., and Z.E. analyzed the data; C.C. wrote the manuscript with contributions of all the authors.

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The authors declare no conflicts of interest.

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