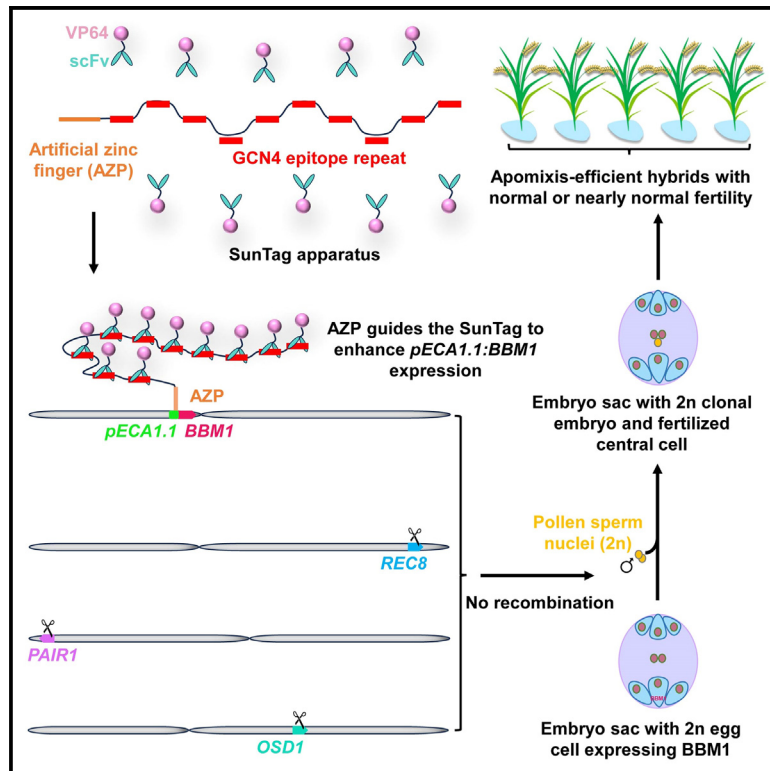


# Engineering high-frequency apomixis with normal seed production in hybrid rice

## Graphical abstract



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## In brief

Biological sciences; Molecular biology;  
Plant biology; Plant genetics

## Highlights

- A SunTag *BBM1* activation system was used to enhance *MiMe-BBM1* apomixis induction in rice
- Apomixis-efficient fertility-normal hybrids were produced via the enhanced apomixis induction
- Hybrid vigor was maintained in the fertility-normal apomictic hybrids



## Article

## Engineering high-frequency apomixis with normal seed production in hybrid rice

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<https://doi.org/10.1016/j.isci.2024.111479>

## SUMMARY

Clonal reproduction through seeds, also termed apomixis, has the potential to revolutionize agriculture by allowing hybrid crops to be clonally propagated. Although apomixis has been introduced into rice through *de novo* engineering in recent years, the poor fertility and low-frequency clonal reproduction of synthetic apomicts hinder the application of apomixis in crop breeding. Here, in elite hybrid rice, we generated many apomicts, which produced clonal progeny with frequencies of > 95.0% and had high even normal fertility through combining enhanced rice BABYBOOM1 (BBM1)-induced parthenogenesis with the simultaneous inactivation of *PAIR1*, *REC8*, and *OSD1*. These synthetic apomicts maintained the agronomic traits of the elite hybrid rice. Our results indicate that fertility-normal hybrids with high penetrance of apomixis can be generated in rice, thereby laying an important foundation for the application of synthetic apomixis in crop breeding.

## INTRODUCTION

Utilization of heterosis plays a fundamental role in crop improvement and is crucial for food security in the world. However, due to genetic recombination, the heterosis phenotype of  $F_1$  hybrids cannot be stably inherited. Apomixis has the potential to fix heterosis by allowing clonal propagation of  $F_1$  hybrids through seeds, therefore holding great promise in agriculture. Apomixis naturally occurs in hundreds of plant species but not in major crops including rice.<sup>1,2</sup> In recent years, synthetic apomixis was achieved in rice through combining *Mitosis instead of Meiosis* (*MiMe*) with the parthenogenesis induced by *MATRILINEAL* (*MTL*) knockout<sup>3</sup> or by egg cell expression of one of the embryonic triggers including rice *BABYBOOM1* (*BBM1*),<sup>4,5</sup> rice *BABYBOOM4* (*BBM4*),<sup>6</sup> and dandelion *PARTHENOGENESIS* (*PAR*).<sup>7</sup> The *MiMe* refers to the simultaneous inactivation of three genes including *PAIR1* or *SPO11-1*, *REC8*, and *OSD1*, which results in conversion from meiosis to mitosis and leads to production of clonal male and female gametes.<sup>8,9</sup> The synthetic apomixis recapitulates the mode of natural apomixis through the formation of clonal egg cell followed by its parthenogenetic development.<sup>3,10</sup>

Although apomixis has been introduced into rice through the above methods, the poor fertility and low-frequency clonal reproduction of apomictic rice hinder the application of apomixis in agriculture. The *MiMe-mtl* apomicts exhibited not only poor fertility (< 5.0%) but also low-frequency clonal reproduction ( $\leq$  9.5%).<sup>11</sup> The *MiMe-BBM4* and *MiMe-PAR* apomicts could

exhibit high fertility but produced clonal progeny at low rates (no more than 3.0% and 68.0, respectively).<sup>6,7</sup> Through the *MiMe-BBM1* strategy, high-frequency clonal progeny (>95%) could be obtained, but remarkably reduced fertilities compared to the wild type were observed in all the *MiMe-BBM1* apomictic rice reported previously.<sup>4,5,12</sup>

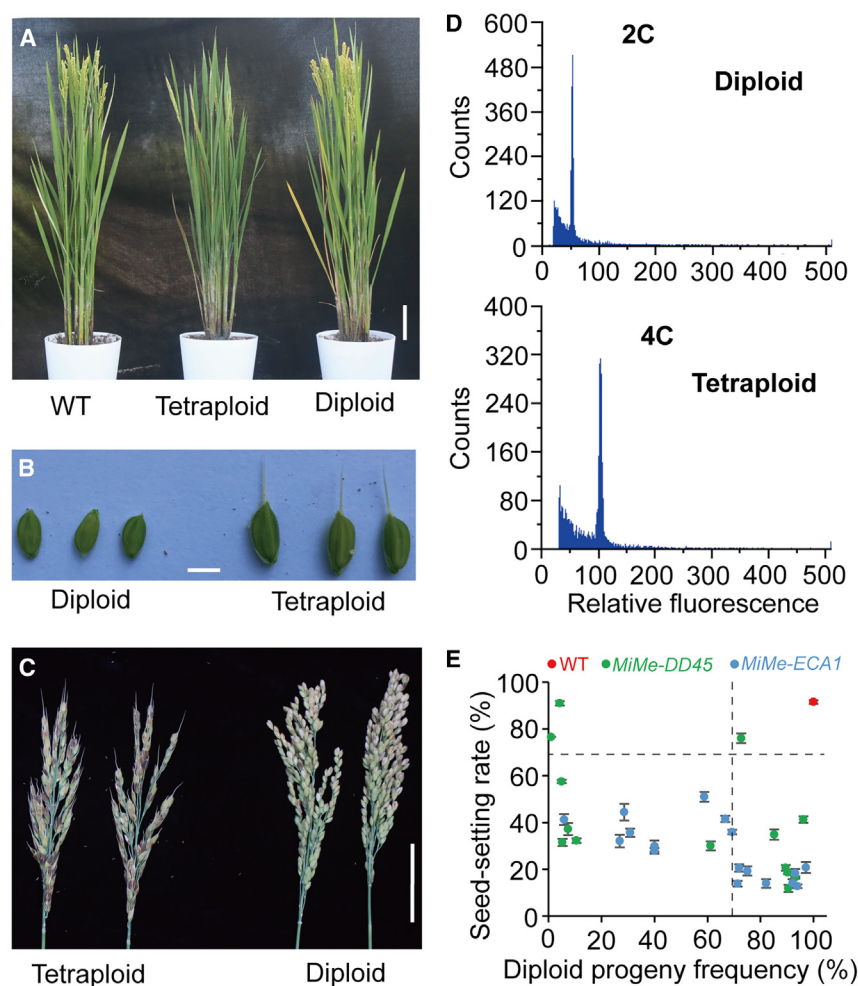
Here, in rice, we further improved the apomixis-inducing efficiency of the *MiMe-BBM1* strategy and generated many apomictic hybrids, which produced clonal progeny with frequencies of 95.0%–100.0% and had fertilities comparable or nearly comparable to the wild type, through combining enhanced *BBM1*-induced parthenogenesis with the simultaneous inactivation of *PAIR1*, *REC8*, and *OSD1*. These results indicate that fertility-normal hybrids with apomixis frequencies of >95.0% can be generated in rice, thereby laying an important foundation for the application of synthetic apomixis in crop breeding.

## RESULTS

## Engineering high-frequency apomixis with high fertility in rice

In recent years, a high-frequency apomixis was achieved in rice through combining *MiMe* with *BBM1*-induced parthenogenesis, but fertilities of the resultant apomicts were remarkably reduced compared to the wild type.<sup>5,12</sup> We conducted a similar study before the publication of this high-frequency apomixis. In our study, a *MiMe*-inducing vector termed *MiMe-Con* was





**Figure 1. Apomixis in XS134 background**

(A) Comparison of the wild type and the progeny (tetraploid and diploid) of apomicts. Scale bar, 10 cm. WT, the wild type. (B) Spikelets of the diploid and tetraploid progeny of apomicts. Scale bar, 0.5 cm. (C) Panicles of the diploid and tetraploid progeny of apomicts. Scale bar, 5 cm. (D) Ploidy analysis of the diploid and tetraploid progeny of apomicts. (E) Diploid frequencies and fertilities in  $T_1$  progeny of the *MiMe-DD45* and *MiMe-ECA1* apomictic lines (see Table S1 for the detailed data). Data for fertility are represented as means  $\pm$  SD. WT, the wild type.

such as larger and awned spikelets, complete sterility or extremely lower fertility, lower tillering, and darker green leaves (Figures 1A–1C). The ploidy discrimination based on phenotype was confirmed by flow cytometry analysis (Figure 1D). In contrast to the *MiMe-Con* *MiMe* plants (*MiMe*-only plants), the apomictic lines (*MiMe-DD45* and *MiMe-ECA1* apomicts in XS134 background) produced 1.0%–97.1% diploid progeny in  $T_1$  generation, and about half of the apomictic lines (7 of the 14 *MiMe-DD45* apomictic lines and 8 of the 17 *MiMe-ECA1* apomictic lines) showed diploid rates of >70.0% (72.7%–96.2% for *MiMe-DD45* and 71.3%–97.1% for *MiMe-ECA1*) in  $T_1$  generation (Figure 1E and Table S1), consistent with the previous report that high-frequency apomixis could be engineered through combining *MiMe* with *BBM1*

constructed to knockout *PAIR1*, *REC8*, and *OSD1* simultaneously through CRISPR/Cas9 (Figure S1A). Then, one rice *BBM1* expression cassette with *Arabidopsis* *DD45* promoter (*pDD45*) or rice *ECA1.1* promoter (*pECA1.1*) was introduced to *MiMe-Con*, generating two apomixis-inducing vectors termed *MiMe-DD45* (with *pDD45:BBM1* expression cassette) and *MiMe-ECA1* (with *pECA1.1:BBM1* expression cassette) (Figures S1B and S1C). *Agrobacterium*-mediated transformation of a *japonica* rice cultivar Xiushui134 (XS134) generated 14 and 17 apomictic lines with *MiMe-DD45* and *MiMe-ECA1*, respectively. Additionally, 13 *MiMe* plants, 3 (plants Cn1, Cn2, and Cn3) of which showed apparently normal fertility, were generated through *Agrobacterium*-mediated transformation of XS134 with *MiMe-Con*. In rice diploid *MiMe* plants, parthenogenesis would result in diploid progeny, which were genetically identical to the mother plants.<sup>4,5</sup> Otherwise, fertilization of the egg cells in *MiMe* plants would double the ploidy at each generation.<sup>4,5</sup> Consistent with the absence of parthenogenesis-inducing element (*BBM1* expression cassette) in *MiMe-Con*, all the progeny of the *MiMe-Con* *MiMe* plants were tetraploids (Table S1), which showed distinct phenotypes from the wild type and the diploid progeny of the apomictic plants,

expression driven by *pDD45* or *pECA1.1*.<sup>5</sup> We also transformed two commercial hybrid cultivars Yongyou4949 (YY4949) and Yongyou1538 (YY1538) with *MiMe-ECA1*, and about 38%–43% of the resultant apomictic lines (8 of the 19 YY4949 apomictic lines and 5 of the 13 YY1538 apomictic lines) showed diploid rates of >70.0% (71.1%–96.0% in YY4949 background and 76.8%–93.5% in YY1538 background) in  $T_1$  generation (Figure S2A and Table S2). All the diploid progeny of the above apomicts were morphologically similar to the wild type (Figures 1A, S2B, and S2C). Except for the diploid offspring, the other progeny of the above apomictic lines were tetraploids. Among the above apomictic lines, two (A14, one *MiMe-DD45* XS134 line and C12, one *MiMe-ECA1* YY4949 line) showed diploid fertilities comparable to the wild type ( $p > 0.05$ ) but produced diploid progeny with frequencies of < 7.0% (4.3% and 6.5%, respectively) in  $T_1$  generation (Figures 1E and S2A; Tables S1 and S2), indicating that *MiMe-BBM1* apomixis does not necessarily reduce fertility. In  $T_1$  generation, besides the 2 fertility-normal apomicts, 6 lines (*MiMe-DD45* lines A3 and A4, and *MiMe-ECA1* lines C13, C19, D4, and D9) of the above apomicts also showed diploid fertilities of >70.0% (76.5%, 75.9%, 83.3%, 77.0%, 72.4%, and 81.3%, respectively), which were about

4.1%–15.5% less fertile than the wild type (Figures 1E and S2A; Tables S1 and S2). 2 of the 6 high-fertile apomictic lines (lines A4 and D4) showed diploid rates of >70.0% (72.7% and 82.2%, respectively) in  $T_1$  generation (Tables S1 and S2), indicating that high-frequency apomixis with high fertility can be engineered with *MiMe-DD45* and *MiMe-ECA1*.

Most of the above apomictic lines showed remarkably reduced diploid fertilities compared to the wild type (Figures 1E and S2A). Incomplete knockout of the *MiMe* genes (*PAIR1*, *REC8*, and *OSD1*) would lead to defective meiotic chromosome segregation,<sup>9,13,14</sup> which would cause complete sterility or severely reduced fertility. Therefore, to explore the reason for the reduced fertility in apomicts, we examined the mutations in the above apomicts and found that except 9 extremely low-fertile apomicts (lines A1, A8, B5, B7, B13, C5, C6, C18, and D13. See Tables S1 and S2 for the data of fertility) with non-frameshift lesion or wild-type allele in *OSD1* locus, all the other apomicts (54 lines) contained frameshift mutations in both the two alleles of each *MiMe* gene (*PAIR1*, *REC8*, and *OSD1*) (see Dataset S1 for the mutations), suggesting that incomplete knockout of *PAIR1*, *REC8*, and *OSD1* is not the underlying reason for the reduced fertilities in most of the above apomicts. Therefore, considering the reduced fertilities in most of the above apomicts, we speculate that *MiMe-BBM1* apomixis, although not necessarily, affects fertility in rice.

### Improvement in rice apomixis induction

Most of the above apomictic lines showed diploid rates of <90.0%, and engineering high-frequency apomixis with high fertility may be challenging in this limited apomixis induction efficiency. To improve apomixis induction efficiency, we utilized SunTag gene activation technology<sup>15,16</sup> to enhance the transgenic *BBM1* expression in egg cells. To utilize the SunTag technology, two artificial zinc finger proteins (AZP1 and AZP2) each recognizing a 19-bp sequence in *pECA1.1* were designed to recruit transcription activating factor VP64 through interaction between AZP1/2-fused tandem GCN4 (AZP1-10 x GCN4 and AZP2-10 x GCN4) and GCN4 antibody fusion protein scFv-sfGFP-VP64. The SunTag expression elements were integrated into *MiMe-ECA1* to generate two vectors, *MiMe-ECA1-AZP1* and *MiMe-ECA1-AZP2*, which used AZP1 and AZP2, respectively, to recognize *pECA1.1* (Figure 2A). We transformed XS134 and YY4949 with *MiMe-ECA1-AZP1*, and in  $T_0$  generation, less than half of the resultant apomictic lines (5 of 11 XS134 apomictic lines, and 6 of 15 YY4949 apomictic lines) showed diploid rates of >70.0% (Table S3), and no high-fertile apomixis with >90.0% diploid rate was observed. In contrast to *MiMe-ECA1* and *MiMe-ECA1-AZP1*, most of the *MiMe-ECA1-AZP2* apomictic lines (12 of 14 XS134 apomictic lines, 10 of 13 YY4949 apomictic lines, and 15 of 18 YY1538 apomictic lines) showed diploid rates of >90.0% (90.1%–100.0%) in  $T_1$  generation (Figure S3 and Table S4), suggesting that *MiMe-ECA1-AZP2* was more efficient than the other vectors (*MiMe-DD45*, *MiMe-ECA1*, and *MiMe-ECA1-AZP1*) in apomixis induction. To test whether the SunTag elements in *MiMe-ECA1-AZP2* promoted *pECA1.1:BBM1* expression, we detected the expression in embryos at 5 days after pollination (DAP) through RT-qPCR (*ECA1.1* shows specific expression in egg cells and em-

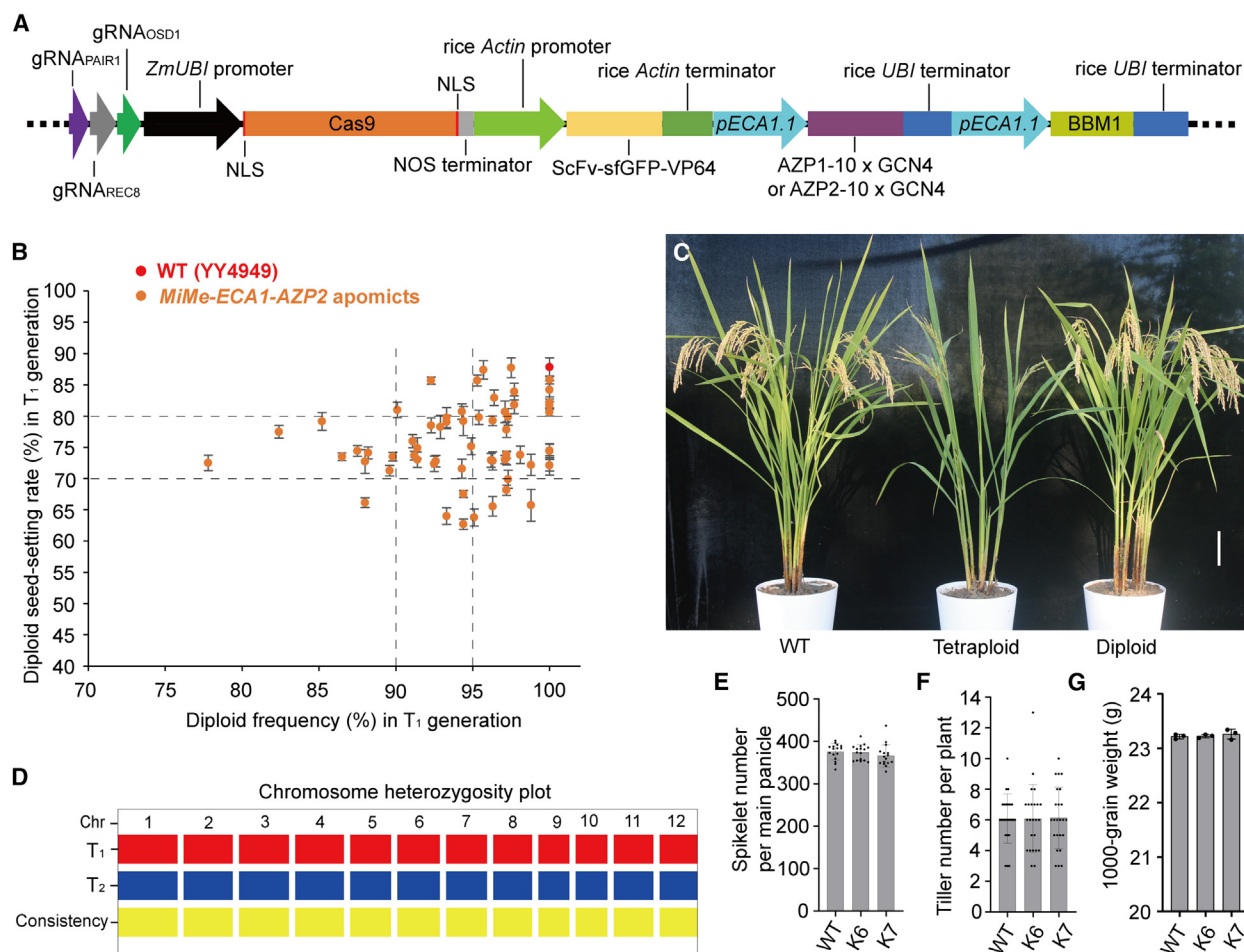
bryos<sup>17,18</sup>), and found that the *pECA1.1:BBM1* expressions in *MiMe-ECA1-AZP2* apomicts were overall stronger than those in *MiMe-ECA1* apomicts (Figure S4).

In  $T_1$  generation, among the above *MiMe-ECA1-AZP2* apomictic hybrids, 3 YY4949 lines (apomictic lines H6, H11, and H12) and 4 YY1538 lines (apomictic lines I1, I2, I3, and I5) produced >80.0% (96.2%, 100.0%, 84.8%, 96.4%, 100.0%, 89.8%, and 96.7%, respectively) diploid progeny and showed diploid fertilities of > 70.0% (72.8%, 78.0%, 83.4%, 83.8%, 82.0%, 73.7%, and 77.3%, respectively), which were only 1.3%–14.6% less fertile than the wild type (Figure S3 and Table S4). The above results indicate that high-frequency apomixis with nearly normal fertility can be generated through *MiMe-ECA1-AZP2*.

### Engineering high-frequency apomixis with normal fertility in hybrid rice

Besides synthetic apomixis, the callus culture-based transgene manipulation may also have negative effects on fertility. The callus culture-based transgene manipulation would induce genome variation, which often reduced fertility, and seed-setting rate fully comparable to the wild type is not a very high-frequency phenomenon in rice transgenic events. Therefore, to explore whether we could engineer high-frequency apomixis with normal fertility, we conducted a large-scale transformation of YY4949 with *MiMe-ECA1-AZP2*, and in  $T_0$  generation, 17 *MiMe* plants (plants K1 to K17) with apparently normal or nearly normal fertility were identified from 307 fertile transgenic events. In  $T_1$  generation, 2 of the 17 plants (plants K6 and K7) produced 95.7%–97.5% diploid progeny which showed seed-setting rates (range: 84.4%–89.7%) comparable to that of the wild type (87.8%, range: 85.2%–90.2%) ( $p > 0.05$ , all spikelets of 8 plants for each material were investigated for the fertility analysis) (Table S5). The diploid progeny of the other high-fertile *MiMe* plants (plants K1 to K5 and K8 to K17) also showed apparently normal fertility, but the diploid frequencies for these *MiMe* plants (plants K1 to K5 and K8 to K17) were < 3.7% in  $T_1$  generation (Table S5). Why the fertility-normal apomicts were prone to show low apomixis rates remains to be investigated. Besides the 17 high-fertile *MiMe* plants (plants K1 to K17), we also harvested seeds from 140 randomly chosen  $T_0$  plants (most of which showed severely reduced fertilities) in the 307 *MiMe-ECA1-AZP2* transgenic events. In  $T_1$  generation, to our surprise, about half of the 140 lines (73 lines) showed high fertilities of more than about 60.0%. We identified 59 apomictic lines (lines K18 to K76) from the 73 transgenic events (Table S5). In  $T_1$  generation, most (49 lines) of the 59 apomictic lines produced >90.0% (90.1%–100.0%) diploid progeny and showed diploid seed-setting rates of 62.1%–86.7% (vs. 85.2%–90.2% in the wild type) (Figure 2B and Table S5). Among the 59 apomictic lines, 10 lines (lines K18, K20, K22, K31, K35, K48, K51, K58, K64, and K65) produced 95.3%–100.0% diploid progeny which showed seed-setting rates of >80.0% (80.7%–86.7%) in  $T_1$  generation (Figure 2B and Table S5). These results indicate that high-frequency apomixis with normal or nearly normal fertility can be efficiently engineered with *MiMe-ECA1-AZP2* in hybrid rice.

We also again transformed YY4949 with *MiMe-ECA1*, and no high-fertile (fertilities of > 60.0%) apomixis with diploid rate



**Figure 2. Apomixis-efficient high-fertile hybrids generated in the large-scale transformation of YY4949 with MiMe-ECA1-AZP2** (A) Schematic representations of MiMe-ECA1-AZP1 and MiMe-ECA1-AZP2. gRNA<sub>PAIR1</sub>, gRNA<sub>REC8</sub>, and gRNA<sub>OSD1</sub>, gRNA expression cassettes for PAIR1, REC8, and OSD1 knockout, respectively. (B) Diploid frequencies and fertilities in T<sub>1</sub> progeny of the high-fertile apomixis-efficient hybrids generated in the large-scale transformation of YY4949 with MiMe-ECA1-AZP2 (see Table S5 for the detailed data). (C) Comparison of the wild type and the progeny (tetraploid and diploid) of the fertility-normal apomicts. Scale bar, 10 cm. (D) Whole-genome sequencing analysis of two K6 apomictic plants using 1 559 769 high-confidence SNPs. Red, heterozygous SNPs in the T<sub>1</sub> plant of K6 apomictic line; dark blue, heterozygous SNPs in the T<sub>2</sub> plant of K6 apomictic line; yellow, identical SNP heterozygosity in the T<sub>1</sub> and T<sub>2</sub> plants; consistency, the consistency of SNP genotype in the T<sub>1</sub> and T<sub>2</sub> plants. (E) Spikelet number per main panicle. Two main tillers per plant were investigated in eight plants of every material. (F) Tiller number per plant. 24 plants per material were investigated. (G) 1000-grain weights of the seeds from the wild type and the fertility-normal apomicts. The agronomic traits were investigated in T<sub>1</sub> generation. WT, the wild type (YY4949). Data for fertility, spikelet number, tiller number, and 1000-grain weight are represented as means  $\pm$  SD.

of >90.0% was observed in 83 fertile MiMe-ECA1 transgenic events. The apomixis efficiencies and fertilities of the above apomictic lines were also investigated in the next generations, and stable diploid rates and fertilities were observed in the above MiMe-DD45, MiMe-ECA1, and MiMe-ECA1-AZP2 apomicts from T<sub>1</sub> to T<sub>3</sub> generations (Tables S6 and S7).

### The apomixis fixed the heterozygous genotype and hybrid vigor in hybrid rice

The F<sub>2</sub> progeny of YY4949 and YY1538 showed obvious phenotypic segregation, whereas all the diploid progeny of the

apomictic hybrids were morphologically similar to the wild type (Figures 2C, S2B, S2C, and S6A) and did not show obvious phenotypic segregation in T<sub>1</sub> and subsequent generations, suggesting fixation of heterozygous genotype. The fixation of heterozygous genotype by MiMe-BBM1 apomixis has been reported by two research works.<sup>4,5</sup> To confirm the fixation of heterozygous genotype in this study, 7 insertion-deletion (InDel) markers (Table S8), which were distributed on different chromosomes and showed heterozygosity in YY4949, were designed. All the 7 InDel markers showed segregation in F<sub>2</sub> progeny of YY4949 but not in diploid progeny of the high-fertile apomictic

hybrids (Figures S5A–S5F), consisting with the fixation of genome heterozygosity by apomixis. We also performed whole-genome sequencing of two diploid plants from different generations ( $T_1$  and  $T_2$  generations) of K6 apomictic line (YY4949 background), and bioinformatic analysis of the sequencing data using 1 559 769 SNPs showed that the two plants were heterozygous at the whole genome and were genetically identical to each other (Figure 2D), confirming the fixation of genome heterozygosity by apomixis.

The fertility-normal apomictic hybrids showed no apparent differences with the wild type in plant height (Figure 2C), spikelet number per main panicle ( $p > 0.05$ ) (Figure 2E), tiller number per plant ( $p > 0.05$ ) (Figure 2F), panicle morphology (Figure S6B) in  $T_1$  and  $T_2$  generations. In addition, no obvious differences in 1000-grain weight and grain shape were observed between the seeds harvested from the wild type and the fertility-normal apomictic hybrids (lines K6 and K7) ( $p > 0.05$ ) (Figures 2G and S6C). The above results suggest that the hybrid vigor was fixed by the high-frequency apomixis in the fertility-normal apomictic hybrid rice.

## DISCUSSION

### Reduced fertility in *MiMe-BBM1* apomicts may result from comprehensive effects of parthenogenesis and transgene manipulation-induced genomic variation

High-frequency apomixis in rice has been achieved through combining *MiMe* with *BBM1* expression driven by *pDD45* or *pECA1.1*, but reduced fertility was observed in all the *MiMe-BBM1* apomicts reported previously.<sup>5</sup> In this study, we also achieved high-frequency apomixis (diploid rates of up to 97.1% in  $T_1$  generation) in rice through the same methods. In addition, we obtained a few fertility-normal apomicts through these methods (*MiMe-DD45* and *MiMe-ECA1*) (although the apomixis efficiencies were very low), indicating that *MiMe-BBM1* apomixis does not necessarily reduce fertility.

Obviously reduced fertility was observed in most of the apomicts in this study. Incomplete knockout of the *MiMe* genes (*PAIR1*, *REC8*, and *OSD1*) would lead to defective meiotic chromosome segregation,<sup>9,13,14</sup> which would result in complete sterility or severely reduced fertility. Although incomplete knockout of *OSD1* was observed in a few apomictic lines with severely reduced fertilities, most of our apomictic lines contained frame-shift mutations in both the two alleles of each *MiMe* gene (see Dataset S1, S2, and S3 for the mutated sequences), suggesting that incomplete knockout of the *MiMe* genes is not the underlying reason for the reduced fertilities in most of the above apomictic lines. Therefore, considering the remarkably reduced fertilities in most of the above apomicts, we speculate that *MiMe-BBM1* apomixis, although not necessarily, affects fertility in rice.

Consistent with a previous report,<sup>3</sup> apparently normal fertility was observed in our *MiMe*-only plants in  $T_0$  generation, suggesting that the reduced fertility in *MiMe-BBM1* apomicts did not result from the transition of meiosis to mitosis induced by simultaneous knockout of *PAIR1*, *REC8*, and *OSD1*. Thus, we infer that *BBM1*-induced parthenogenesis but not *Mitosis instead of Meiosis (MiMe)* in the *MiMe-BBM1* apomicts, although not necessarily, reduces fertility in rice.

The start time of parthenogenesis development may have effects on the fertility and apomixis efficiency. The position and copy number of transfer DNA insertion in genome should affect the expression timing and level of the transgenic *BBM1*, which may lead to different start time of egg cell parthenogenetic development in different apomictic lines, resulting in the variation of fertility among the apomictic lines.

In addition to the above fertility-reduced factors (parthenogenesis and incomplete knockout of *OSD1*), the callus culture-based transgene manipulation should also have effects on fertility. The callus culture-based transgene manipulation would induce genome variation, which often reduced fertility, and seed-setting rate fully comparable to the wild type is not a very high-frequency phenomenon in rice transgenic events. Therefore, we speculate that the reduced fertility in *MiMe-BBM1* apomictic rice result from the comprehensive effects of parthenogenesis and transgene manipulation-induced genomic variation (as well as incomplete knockout of *OSD1* in some apomicts).

### High-frequency *MiMe-BBM1* apomixis does not necessarily reduce fertility

To improve the apomixis induction efficiency, we integrated the SunTag gene activation system<sup>15,16</sup> into *MiMe-ECA1* vector to enhance transgenic *BBM1* expression, which brought a more efficient apomixis-inducing vector (*MiMe-ECA1-AZP2*). We generated many rice plants with increased penetrance of apomixis (clonal diploid rates of up to 100.0%) through *MiMe-ECA1-AZP2*. This result suggests that enhancing the transgenic *BBM1* expression can improve the apomixis-inducing efficiency.

The improvement in apomixis induction through *MiMe-ECA1-AZP2* seemingly did not reduce the overall level of fertility in the resultant apomictic plants, and many apomictic hybrids, which produced clonal progeny with frequencies of  $> 95.0\%$  and had normal or nearly normal fertilities, were obtained through *MiMe-ECA1-AZP2*. This result indicates that high-frequency *MiMe-BBM1* apomixis does not necessarily reduce fertility.

### An outlook on how to realize application of apomixis in crop breeding

Apomixis has the potential to revolutionize agriculture by allowing hybrid crops to be clonally propagated. However, poor fertility and low apomixis efficiency hinder the application of apomixis in crop agriculture. In this study, through an improved apomixis-inducing vector termed *MiMe-ECA1-AZP2*, we constructed many apomictic hybrids which produced clonal progeny with frequencies of 95.0%–100.0% and had normal or nearly normal fertilities (up to seed-setting rate of 89.7%). This result indicates that fertility-normal hybrids with apomixis frequencies of  $> 95.0\%$  can be generated in rice, thereby laying an important foundation for the application of apomixis in rice breeding. However, to realize the application of apomixis in rice breeding, there are some questions remaining to be solved. First, the apomictic rice plants here and reported previously are transgenic products, and massive application of transgenic rice is not permitted in major rice-farming countries (China, India, Bangladesh, etc.). Therefore, cultivation of transgene-free or T-DNA-free apomictic rice should be an important direction of effort. The advance in genome editing technology may provide solutions for cultivation

of T-DNA-free apomictic rice. Second, somatic variations often occur during the process of callus culture-based transgene manipulation, which may reduce the fertility and grain yield. Transgenic crop breeding often requires hybridization (with the wild type or other normal materials) and subsequent genetic segregation to discard the adverse somatic variations. However, due to the clonal mode of reproduction, apomictic rice cannot undergo sexual hybridization to remove the adverse variation. Thus, we propose that a switch of apomictic reproduction should be developed to enable apomictic rice to get rid of the adverse somatic variation and transgenic elements through sexual reproduction.

### Limitations of this study

This study provides an effective method to engineer high-frequency apomixis with nearly normal fertility and demonstrates that fertility-normal hybrids with apomixis frequencies of >95.0% can be generated in rice, thereby laying an important foundation for the application of apomixis in crop breeding. However, due to the transgenic nature of the synthetic apomixis here and reported previously, apomixis may not be permitted to be used in rice production activities in major rice-farming countries. Therefore, in the future, cultivation of transgene-free or T-DNA-free apomictic rice should be an important direction of effort.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to the lead contact, Chunbo Miao ([chunbomiao@163.com](mailto:chunbomiao@163.com)).

#### Materials availability

All the rice materials in this study are available to request from the corresponding author Chunbo Miao ([chunbomiao@163.com](mailto:chunbomiao@163.com)).

#### Data and code availability

- The raw data of the whole-genome sequencing have been deposited in the Genome Sequence Archive<sup>19</sup> in National Genomics Data Center<sup>20</sup> (China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences) and are publicly accessible at <https://bigd.big.ac.cn/gsa/browse/CRA018812>.
- The other data reported in this paper will be shared by the [lead contact](#) upon request.
- This paper does not report the original code.
- Any additional information required to analyze the data reported in this paper is available from the [lead contact](#) upon request.

### ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of Henan in China (grant no. 222301420105) and the National Natural Science Foundation of China (grant no. 32472137).

### AUTHOR CONTRIBUTIONS

C.M. designed the research. Q.Z. gave important suggestions and supports for the research. M.S. and C.M. constructed the apomictic materials, and C.M., M.S., H.H., F.L., and D.L. conducted the phenotype analysis together. M.S. conducted the flow cytometry analysis. Z.C. conducted the bioinformatic analysis of whole-genome sequencing data. The other assays were conducted by C.M., H.H., and F.L. together. The manuscript was prepared by C.M., and

the other authors provided useful suggestions. All the authors read and approved the final manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2024.111479>.

Received: May 31, 2024

Revised: July 18, 2024

Accepted: November 22, 2024

Published: November 26, 2024

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
<i>Agrobacterium tumefaciens</i> used in rice transformation	Shanghai Weidi	AC1010
<b>Chemicals, peptides, and recombinant proteins</b>		
DAPI	Roche	10236276001
Hygromycin	PhytoTech	H397
DNase I	Invitrogen	18068015
EvaGreen	Biotium	31000
<b>Critical commercial assays</b>		
Seamless cloning kit for vector construction	TransGen	CU201
2×EasyTaq® PCR SuperMix	Transgen	AS111
Reverse transcription assay kit	Invitrogen	18080044
RNA extraction kit	Invitrogen	12183555
<b>Deposited data</b>		
Whole-genome sequencing data generated in this study	This paper	<a href="https://bigd.big.ac.cn/gsa/browse/CRA018812">https://bigd.big.ac.cn/gsa/browse/CRA018812</a>
<b>Recombinant DNA</b>		
Vector <i>MiMe-Con</i>	This paper	N/A
Vector <i>MiMe-DD45</i>	This paper	N/A
Vector <i>MiMe-ECA1</i>	This paper	N/A
Vector <i>MiMe-ECA1-AZP1</i>	This paper	N/A
Vector <i>MiMe-ECA1-AZP2</i>	This paper	N/A
<b>Software and algorithms</b>		
GraphPad Prism v8	GraphPad	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
Rice variety XS134	Henan Agricultural University	N/A
Rice variety YY4949	Wuhan GoldCrop Biotech	N/A
Rice variety YY1538	Jiangxi Xingan Seed Industry	N/A

### EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

#### Plant cultivation

All the rice plants in this study were cultivated in paddy field at Yuanyang county (Henan province, China) in the crop seasons (May to October) of the years 2020 to 2023 and at Ledong county (Hainan province, China) from December to May in the years 2021 to 2023.

### METHOD DETAILS

#### Plant material construction

A CRISPR/Cas9 vector (termed *MiMe-Con*) with pCambia1300 backbone was constructed to knockout *PAIR1*, *REC8*, and *OSD1* simultaneously (Figure S1A; see Table S9 for the Cas9 targets; see Data S1 for the sequences of the gRNA expression cassettes), and then the *pDD45:BBM1* or *pECA1.1:BBM1* expression cassette (see Data S2 for the sequences) was integrated into *MiMe-Con* to generate *MiMe-DD45* or *MiMe-ECA1* (Figures S1B and S1C), respectively. Two artificial zinc finger proteins (AZP1 and AZP2) each recognizing a 19-bp sequence (5'-ctctctgcctcctctcctg-3' and 5'-ccctttccacacgctacg-3', respectively) in *pECA1.1* were designed according to published literatures,<sup>21,22</sup> and *pECA1.1:AZP1-10 x GCN4* and *pECA1.1:AZP2-10 x GCN4* expression cassettes (see Data S3 for the sequences) were constructed and integrated into *MiMe-ECA1* to generate two vectors termed *MiMe-AZP1* (with *pECA1.1:AZP1-10 x GCN4* expression cassette) and *MiMe-AZP2* (with *pECA1.1:AZP2-10 x GCN4* expression cassette). Then, the

scFv-sfGFP-VP64 expression cassette (*pActin:scFv-sfGFP-VP64*) (see [Data S4](#) for the sequence) was integrated into *MiMe-AZP1* and *MiMe-AZP2* to generate *MiMe-ECA1-AZP1* and *MiMe-ECA1-AZP2*, respectively ([Figure 2A](#)).

*MiMe-Con*, *MiMe-DD45*, *MiMe-ECA1*, *MiMe-ECA1-AZP1*, and *MiMe-ECA1-AZP2* were transformed into XS134, YY4949, and YY1538 through *Agrobacterium*-mediated method, and *MiMe* plants were identified from the transgenic rice through sequencing the Cas9 target sites of *PAIR1*, *REC8*, and *OSD1* (see [Dataset S1](#), [S2](#), and [S3](#) for the detected mutations). All the materials were grown in paddy field for analysis.

### Rice transformation

All apomixis-inducing vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 using the freezing-heat shock method. 50 mg/L hygromycin (PhytoTech, Cat. No. H397) was used to select transgenic calli and plants during the *Agrobacterium*-mediated transformation<sup>23</sup> of XS134, YY1538, and YY4949.

### Apomixis efficiency analysis

The diploid progeny of *MiMe-BBM1* *MiMe* plants, which were clonal to the mother plants, result from apomictic reproduction.<sup>4,5</sup> Whereas the fertilization of the egg cells in diploid *MiMe* plants produces tetraploid progeny.<sup>4,5</sup> The tetraploid progeny showed distinct phenotypes to diploid plants, such as larger and awned spikelets, complete sterility or severely lower fertility, lower tillering, and darker green leaves ([Figures 1A–1C](#), [2C](#), [S2B](#), and [S2C](#)), which allowed easy discrimination of tetraploids from diploids. The apomixis efficiency was represented as diploid frequency in the progeny of *MiMe-BBM1* apomictic plants.

### Flow cytometry analysis

The ploidy characterization based on phenotype was confirmed by flow cytometry analysis, which was conducted as follow. Young tender leaves from the wild type and *MiMe-BBM1* plants were collected and chopped, and nuclei were prepared from chopped leaves.<sup>24</sup> The DAPI-stained nuclei suspensions were analyzed with CyFlow Space Flow Cytometer (System Partec, Muenster, Germany).

### RT-qPCR

Total RNA was extracted from embryos at 5 DAP using Invitrogen TRIzol<sup>TM</sup> plus RNA extraction kit (Invitrogen, Cat. No. 12183555). Before reverse transcription, the total RNA was treated with DNase I (Invitrogen, Cat. No. 18068015) to remove the possible genomic DNA contamination. Reverse transcription was performed using the SuperScript<sup>®</sup> III Reverse Transcriptase (Invitrogen, Cat. No. 18080044). Real-time PCR analyses were performed using the Bio-Rad CFX96 real-time PCR instrument and EvaGreen (Biotium, Cat. No. 31000). The PCR was conducted with primers ([Table S10](#)) specific for *Ubiquitin* (Os03g0234200) and *pECA1:BBM1* but not endogenous *BBM1* (to detect *pECA1:BBM1* expression specifically, the upstream primer was designed to span the 5'-UTR and coding sequence of *pECA1:BBM1*).

### Agronomic analysis

The agronomic traits were investigated in the paddy field of Yuanyang (Henan province, China). All spikelets in three to eight randomly selected plants of each material were investigated for fertility analysis.

### Genetic segregation analysis of the wild type and apomictic rice with InDel markers

Seven InDel markers ([Table S8](#)), which showed polymorphism in YY4949, were designed according to the sequence variation between *japonica* and *indica* rice. The genetic segregation of the InDel markers was analyzed in F<sub>2</sub> progeny of YY4949 and apomictic populations in T<sub>1</sub> generation.

### Whole-genome sequencing analysis

The 150-bp paired-end reads were generated by Illumina HiSeq2500 resulting in sequence data with approximately 80-fold coverage for each sample. NGSQCtoolkit v2.3.3<sup>25</sup> was used to filter the raw paired-end reads to create clean data. The software Bowtie2 v2.5.2<sup>26</sup> was used to align the clean reads of each plant to rice reference genome (IRGSP-1.0) with parameters "-I 200 -X 1000 -score-min L, 0, -0.6" and "-p 16". We used SAMtools and BCFtools<sup>27</sup> to remove reads that could be mapped to different genomic positions. Then, the unique mapped paired-end reads were used in SNP calling, and a set of 1 559 769 high-confidence SNPs (all in heterozygous state in the two plants) were identified in each plant. The window size (the number of consecutive SNPs in a window) was 0.1kb, and a recombination map was constructed for each chromosome.<sup>11</sup>

### QUANTIFICATION AND STATISTICAL ANALYSIS

The plant number in each statistical analysis was indicated in the Supplemental Tables. GraphPad Prism v8 was used in the statistical analysis. *P*-values were derived from two-sided T-test with equal variance.