Cell Research (2016) 26:1242-1254.

www.nature.com/cr

ORIGINAL ARTICLE

Turning rice meiosis into mitosis

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Introduction of clonal reproduction through seeds (apomixis) in crops has the potential to revolutionize agriculture by allowing self-propagation of any elite variety, in particular F1 hybrids. In the sexual model plant *Arabidopsis thaliana* synthetic clonal reproduction through seeds can be artificially implemented by (i) combining three mutations to turn meiosis into mitosis (*MiMe*) and (ii) crossing the obtained clonal gametes with a line expressing modified CENH3 and whose genome is eliminated in the zygote. Here we show that additional combinations of mutations can turn *Arabidopsis* meiosis into mitosis and that a combination of three mutations in rice (*Oryza sativa*) efficiently turns meiosis into mitosis, leading to the production of male and female clonal diploid gametes in this major crop. Successful implementation of the *MiMe* technology in the phylogenetically distant eudicot *Arabidopsis* and monocot rice opens doors for its application to any flowering plant and paves the way for introducing apomixis in crop species.

***Keywords*:** meiosis; mitosis; apomixis; rice; *MiMe*

Cell Research (2016) **26**:1242-1254. doi:10.1038/cr.2016.117; published online 21 October 2016

Introduction

Asian rice (*Oryza sativa* L.), the first cereal of human consumption, is the staple food for more than half of the mankind. Current rice production (738 million tons of paddy rice over 160 million hectares) has to rise by more than 20% in the next 20 years [1] on the same arable land surface, to meet the demand of an increasing world population mainly residing in rice-eating countries. Enhancing yield potential, limiting inputs and enabling adaptation to unstable biotic and abiotic environments are current breeding targets. Due to the autogamous mode of reproduction, rice varieties are generally pure lines. However, occurrence of heterosis in rice hybrids that translates into a 15%-20% increase in yield potential [2, 3] prompted the development of F1 hybrids. Hybrid rice has been successfully deployed in India, Indonesia, Vietnam, the Philippines, and in China it is grown in more than 50% of the rice-grown areas [4]. However, the seed cost and the necessity to buy new seeds every year prevent its large adoption by subsistence farmers [5]. Creating hybrids that can breed true through a clonal mode of reproduction would result in major agricultural benefits for many crops, including rice, allowing the full harnessing of hybrid vigor [6].

Apomixis is an asexual reproductive process through seeds that bypasses meiosis and fertilization, to produce offspring genetically identical to the mother [6]. Apomixis occurs in over 400 angiosperm species and is notably common in the *Poacae* family, but is absent from the most important crops [7]. Genetic analyses of the inheritance of apomixis in several species including the cereal and grass relatives of the genera *Tripsacum*, *Pennisetum*, *Panicum*, *Bracchiaria* and *Paspalum* have identified a single-chromosome segment that can induce apomixis (reviewed in [8]). However, attempts to transfer this chromosomal segment to their respective cultivated relatives have been unsuccessful [9, 10]. The existence of apomictic relatives in cereals and grasses has prompted the screening of wild rice relatives but no natural source of apomixis was discovered [11, 12]. One possible alternative to create apomixis in crop plants, and notably in rice, is to *de novo* engineer clonal reproduction by targeted modification of the sexual reproduction mode, which has been shown to be possible in *Arabidopsis* [13] (Supplementary information, Figure S1).

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Received 3 June 2016; revised 9 August 2016; accepted 30 August 2016; published online 21 October 2016

The first component of apomixis is apomeiosis, which is the conversion of the meiotic division into a mitotic-like division, leading to the formation of functional, diploid clonal gametes. Several meiotic mutants have been identified in *Arabidopsis*, maize or rice that produce apomeiosis-derived gametes [14]. However, most of these mutations lead to almost complete male and female sterility, and the production of unreduced gametes occurs at a low frequency (e.g., [15]). One exception is the *Arabidopsis MiMe* (*Mitosis instead of Meiosis*) genotype, in which meiosis is turned into a mitotic-like division, associated with both high fertility and production of clonal diploid gametes at a very high frequency (virtually 100%) [16, 17]. *MiMe* is the combination of mutations in three genes (*SPO11-1*, *REC8* and *OSD1*), each mutation impairing one of the three main processes that distinguish meiosis from mitosis. First, *spo11-1* abolishes meiotic recombination [18]. Second, the mutation of *REC8* causes the separation of sister chromatids at first meiotic division, instead of the distribution of homologous chromosomes [19]. Last, *osd1* causes the skipping of the second meiotic division [16]. Therefore, meiosis in *MiMe* occurs without recombination and distributes sister chromatids in a single round of division, mimicking a mitotic division [16]. This results in the production of clonal male and female gametes, but leads to doubling of ploidy at each generation when self-fertilized. Crossing a *MiMe* plant as male or female with a line whose genome is eliminated following fertilization (lines expressing modified CENH3) leads to the production of clonal offspring [13, 20].

The original *MiMe* genotype is a triple mutant *Atspo11-1 Atrec8 osd1* [16]. To facilitate the transfer of the *MiMe* technology to crops, it would be useful to have more potential target genes, notably when the mutant resource is limited or when the genes are represented by several paralogs. For *AtREC8*, there is currently no alternative as the single other gene known to be required for monopolar orientation of kinetochores is *SCC3*, which is also expressed mitotically and thus the corresponding mutation would affect regular development [19]. Two alternatives to *osd1* can be used to create *MiMe*, *tam* and *TDMΔD* [17, 21]. Finally, besides *AtSPO11-1*, several genes have been shown to be essential for initiation of meiotic recombination through DNA double-strand break formation (*AtSPO11-2*, *PRD1*, *PRD2*, *PRD3*/*PAIR1*, *DFO* and *MTOPVIB*) [22-26], but are not yet tested to create the MiMe phenotype.

Given the potential interest in developing apomictic plants and establishing the *MiMe* genotype in crops, the objective of this study was three-fold: (i) extend the catalog of genes that can be mutated to create the MiMe phenotype; (ii) identify the ortholog of the key *OSD1* gene in rice; (iii) determine whether one could establish the MiMe phenotype in rice.

Results

Several mutant combinations give rise to the MiMe phenotype in Arabidopsis

To test the ability of other mutations than *spo11-1* to generate *MiMe*, we constructed triple mutants *prd1 Atrec8 osd1*, *prd2 Atrec8 osd1* and *prd3 Atrec8 osd1* and analyzed their meiotic behavior (Figure 1). In wild type, five bivalents align at metaphase I (Figure 1A), and segregate into two groups of five chromosomes at anaphase I (Figure 1B). After a short interkinesis (Figure 1C), the five pairs of chromatids align on the two metaphase II plates (Figure 1D). The sister chromatids segregate at anaphase II (Figure 1E), giving rise to four spores each containing five chromatids (Figure 1F). In each of the triple mutants, meiosis was modified toward a mitotic-like division: ten univalents aligned on the metaphase I plate (Figure 1G, 1J and 1M) and 10 pairs of sister chromatids segregated evenly at anaphase I (Figure 1H, 1K and 1N). No second division occurred, resulting in the production of two spores each containing 10 chromatids (Figure 1I, 1L and 1O). Thus in this series of triple mutants, meiosis recapitulated the MiMe phenotype. It is likely that mutations in any of the genes required for double-strand break formation, in combination with *osd1* and *Atrec8*, can cause the MiMe phenotype, enlarging the list of genes than can be targeted.

Identification of OsOSD1

There is one *OSD1* homologue in the *Arabidopsis* genome, *UVI4*. Mutation of *UVI4* affects the somatic cell cycle but not meiosis [27]. These two genes originated from a whole-genome duplication that is shared by all *Brassicaceae* [28]. Accordingly, two genes representative of this family are typically found in *Brassicaceae* species and sequence similarity analysis clearly distinguishes the OSD1 proteins from the UVI4 proteins (Figure 2). In other eudicots, many species have only one representative of the gene family, and when two representatives are present they result from a recent whole-genome duplication [28] and are not shared with the closest diploid species analyzed (see, for example, GM *Glycine max* or ME *Manihot esculenta* in Figure 2). In *Poaceae*, an independent whole-genome duplication [28] led to two subgroups of genes, with each species having at least one representative of both (e.g., OS *Oryza sativa*, HV *Hordeum vulgare*, ZM *Zea mays* in Figure 2). This raised the possibility that these two *Poaceae* gene families could

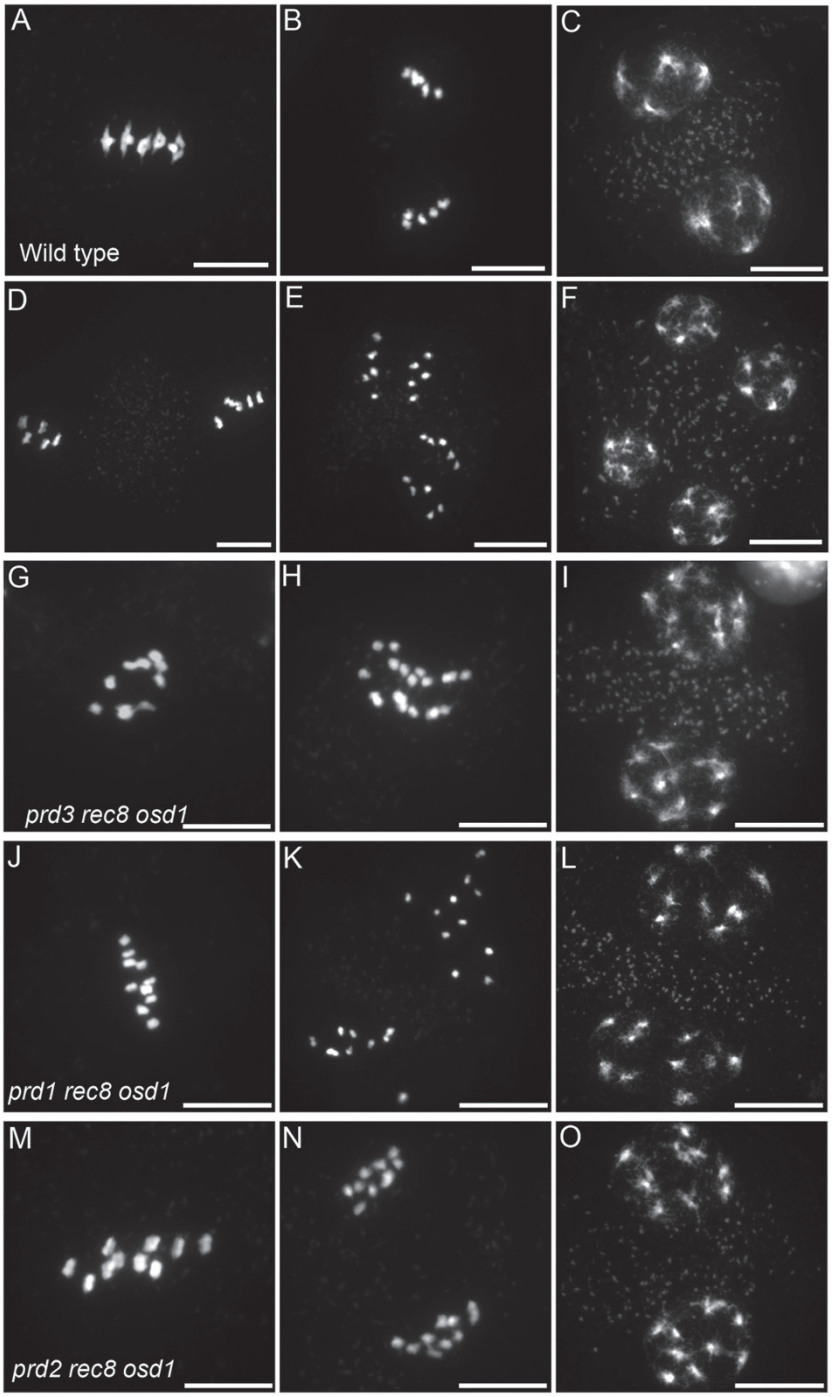

Figure 1 Chromosome spreads of male meiosis in wild-type *Arabidopsis* and *Arabidopsis MiMe* genotypes. **(A**-**F)** Wild type. **(A)** Metaphase I with five aligned bivalents. **(B)** Anaphase I. **(C)** Telophase I. **(D)** Metaphase II. **(E)** Anaphase II. **(F)** Telophase II. **(G**-**I)** *prd3 rec8 osd1* triple mutant (*n* = 17). **(J**-**L)** *prd1 rec8 osd1* triple mutant (*n* = 21). **(M**-**O)** *prd2 rec8 osd1* triple mutant (*n* = 27). **(G**, **J**, **M)** Metaphase I with 10 aligned univalents. **(H**, **K**, **N)** Anaphase I with segregation of 10 pairs of chromatids. **(I**, **L**, **O)** Telophase. Scale bar = 10 μm.

represent *OSD1* and *UVI4* functional homologues, even though they originated from distinct duplications from their *Brassicaceae* counterparts. With the aim of exploring the function of these genes in *Poaceae*, we searched

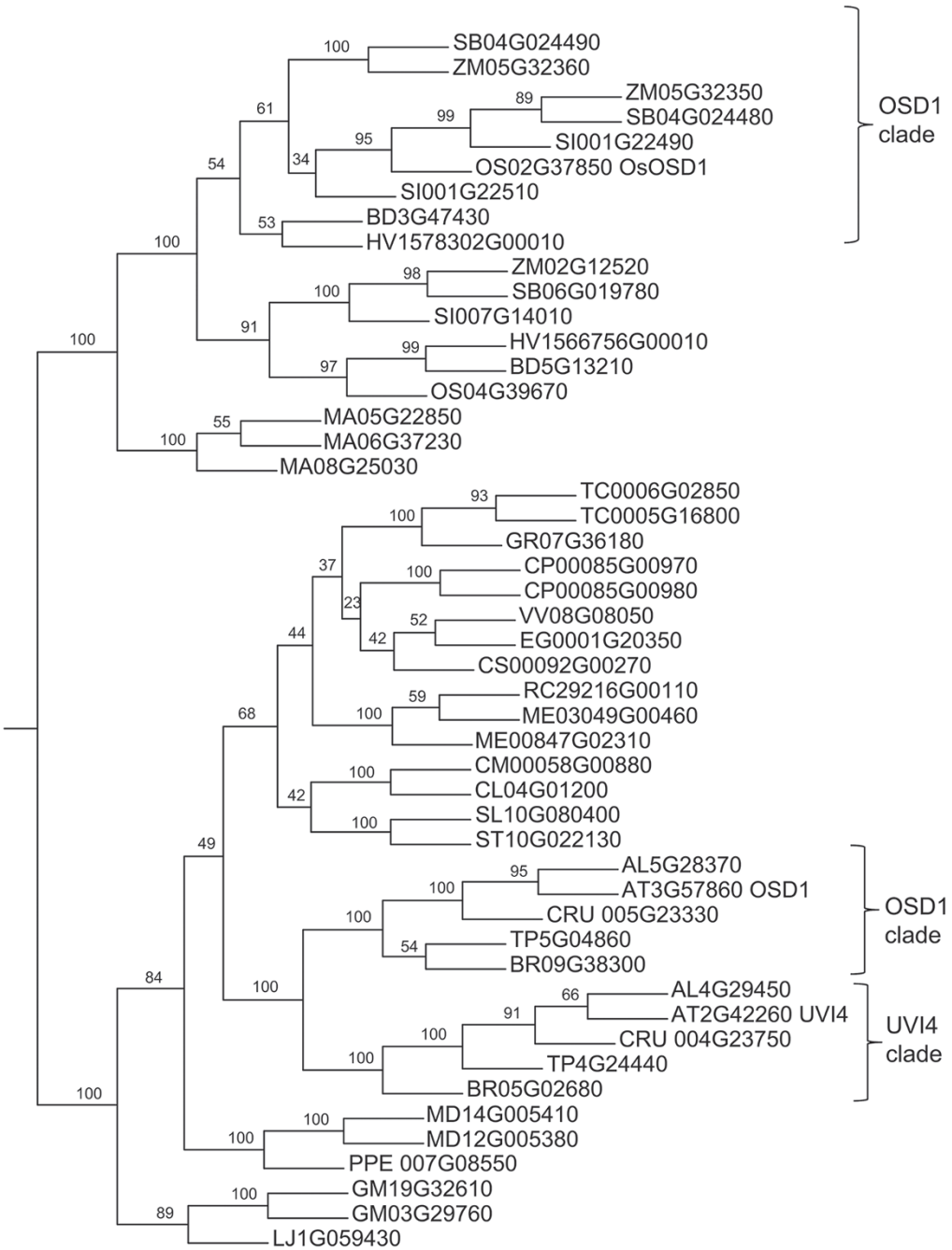

Figure 2 Phylogram of *OSD1* and *UVI4* homologues from flowering plants. Consensus tree of OSD1 and UVI4 protein family inferred from a Muscle alignment. Analyses were performed using the MPI Bioinformatic Toolkit [50], Muscle and Phylip-neighbor with default parameters, enabling boostrapping with 100 replicates. The numbers of forks indicate the number of times the group consisting of the species that are to the right of that fork occurred among the trees, out of 100 trees. AT, *Arabidopsis thaliana*; AL, *Arabidopsis lyrata*; BD, *Brachypodium distachyon*; BR, *Brassica rapa* (turnip mustard); CL, *Citrullus lanatus* (water melon); CM, *Cucumis melo* (muskmelon); CP, *Carica papaya* (papaya); CRU, *Capsella rubella*; CS, *Citrus sinensis* (orange); EG, *Eucalyptus grandis* (eucalyptus); GM, *Glycine max* (soybean); GR, *Gossypium raimondii* (cotton); HV, *Hordeum vulgare* (barley); LJ, *Lotus japonicus*; MA, *Musa acuminata* (banana); MD, *Malus domestica* (apple); ME, *Manihot esculenta* (cassava); OS, *Oryza sativa* (rice); PPE *Prunus persica* (peach); RC *Ricinus communis* (castor bean plant); SB, *Sorghum bicolor* (sorghum); SI, *Setaria italica* (foxtail millet); SL *Solanum lycopersicum* (tomato); ST, *Solanum tuberosum* (potato); TC, *Theobroma cacao* (cacao); VV, *Vitis vinifera* (grape vine); ZM, *Zea mays* (maize).

for mutants in the international rice collections. No insertion was identified in *Os04g39670*, hindering the study of this gene in the present work. However, three insertions were identified in *Os02g37850*. These three insertions, one T-DNA (AMBA12) and two *Tos17* inserts (AMQF10 and ALJA10), are in the second intron (Supplementary information, Figure S2).

Because of omission of the second meiotic division, *Arabidopsis osd1* produces diploid male and female gametes and its self-fertilization generates tetraploid progeny. To address the function of Os02g37850, we used flow cytometry to determine the ploidy of the progeny that are homozygous for the AMBA12, AMQF10 and ALJA10 mutations. This revealed the presence of 100% (n = 82), 37% (n = 27) and 5% (n = 20) of tetraploid plants in the respective progeny, the rest being diploid. This suggests that mutations of Os02g37850 can provoke the production of male and female diploid gametes. AMBA12 appears to be a null mutant, while AMQF10 and ALJA10 appear partially affected. Consistently, RT-PCR experiments showed that the *OSD1* spliced mRNA is barely detectable in AMBA12 homozygotes, and is only reduced in abundance in AMQF10 (Supplementary information, Figure S3). We then compared meiosis in wild type and AMBA12. First, observation of male meiotic products revealed the presence of 100% dyads (n = 500) in AMBA12 homozygotes, instead of tetrads in wild type (Figure 3), showing that a single meiotic division occurs in AMBA12. In AMQF10, a mixture of dyads (29%) and tetrads were observed (n = 130), in accordance with the mixed ploidy in offspring of this mutant. Chromosome spreads showed that the first meiotic division in AMBA12 was indistinguishable from wild type (Figure 4), suggesting that crossover formation and homologous chromosome segregation were unaffected, but no second division took place. Thus, a single

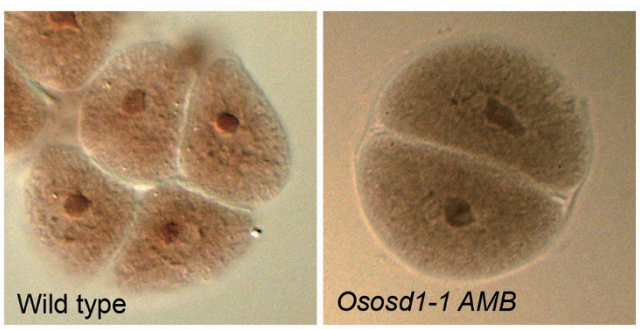

Figure 3 Male meiotic products in wild-type rice and *Ososd1*. Fresh anthers squashed in acetocarmine. **(A)** Tetrads of spores in wild type. **(B)** Dyad of spores in *Ososd1-1* (n = 500).

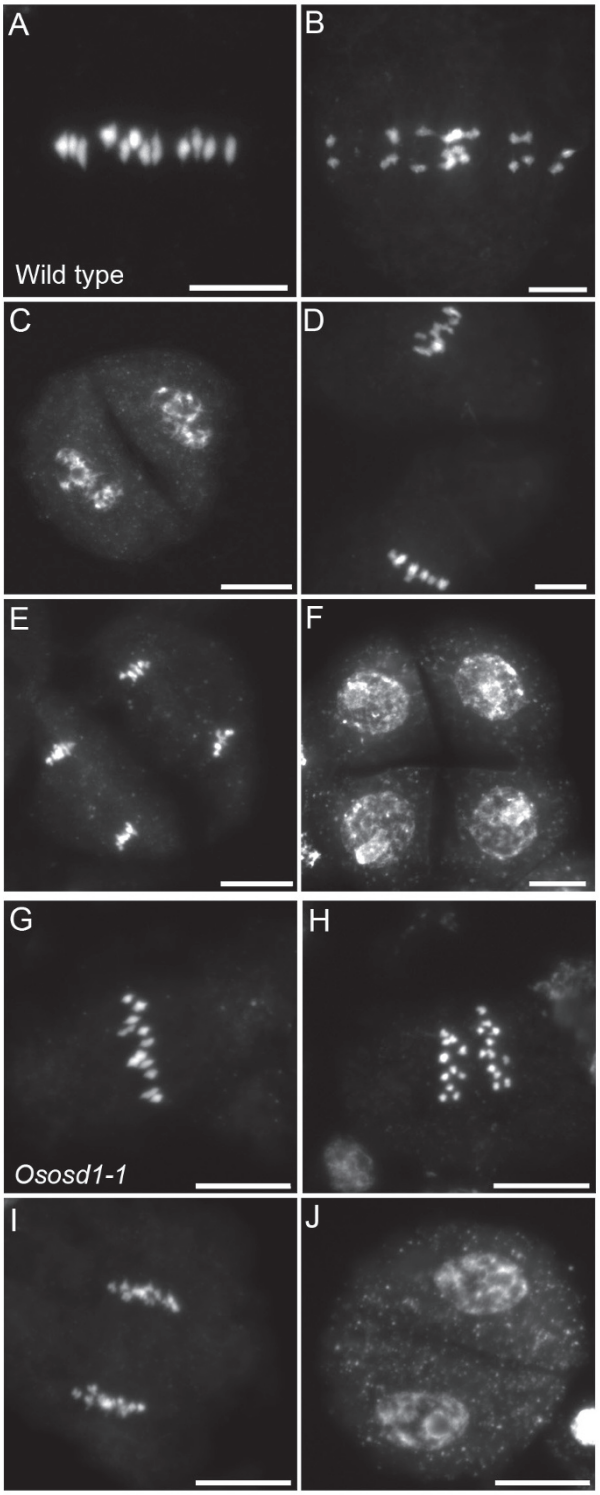

Figure 4 Chromosome spreads of male meiosis in wild-type rice and *Ososd1*. **(A-F)** Wild type. **(A)** Metaphase I with 12 aligned bivalents. **(B)** Anaphase I. **(C)** Telophase I. **(D)** Metaphase II. **(E)** Anaphase II. **(F)** Telophase II. **(G-H)** *Ososd1-1* (n = 62). **(G)** Metaphase I with 12 aligned bivalents. **(H)** Anaphase I. **(I)** Late anaphase I. **(J)** Telophase I. No second division was observed. Scale bar = 10 μm.

first meiotic division occurred in AMBA12. In summary, disruption of *Os02g37850* recapitulated the *Arabidopsis osd1* meiotic defect: the omission of the second meiotic division which leads to the production of functional diploid gametes. We therefore named Os02g37850 *OsOSD1* and AMBA12 *Ososd1-1*.

Frequencies of diploid gametes in Ososd1-1 mutant

To directly determine the ploidy of male gametes in the *Ososd1-1* mutant, we examined the DNA content of nuclei isolated from mature pollen grains with flow cytometry (Figure 5). Pollen nuclei isolated from *OsOSD1*$^{+/+}$ (n = 5) and *OsOSD1*$^{+/-}$ plants (n = 6) exhibited the expected haploid DNA content (Figure 5A and 5C), which was half of the DNA content in leaf nuclei (Figure 5B). In contrast, pollen nuclei isolated from *Ososd1*$^{-/-}$ plants (n = 3) formed a unique peak corresponding to a diploid DNA content. This indicates that all pollen grains produced by *Ososd1* mutants are diploid. We then examined the female meiosis product of *Ososd1*$^{-/-}$ plants by fertilizing them with wild-type pollen. Among 112 seeds obtained, 104 were shrunken and unable to germinate, whereas 8 developed normally and germinated. The obtained eight offspring were diploid and heterozygous for the *Ososd1-1* mutation, indicating they were the result of the fertilization of haploid female gametes produced by *Ososd1-1*. To recover more offspring, we repeated the experiment, by rescuing the embryo at 5 days post fertilization and transferring the resulting plants in soil. Among 47 successfully hybridized flowers, 40 exhibited early signs of abnormal development, including absence of albumen, and developed into triploid plants after embryo rescue. The seven remaining plants, derived from normally developed seeds, were diploid and *Ososd1*$^{+/-}$. Altogether, these results show that ~91% of female gametes produced by *Ososd1-1* were diploid and ~9% (15/159) were haploid. This is reminiscent of the effect of the *osd1* mutation in *Arabidopsis*, in which ~100% of male gametes and ~85% of female gametes are diploid [16]. However, in contrast to the situation in *Arabidopsis*, seeds resulting from the fertilization of a haploid female gamete by a diploid male gamete in rice are not viable and require embryo rescue, due to abnormal endosperm development [29]. The identification of the *OSD1* ortholog in rice and the high frequency of diploid gametes produced by the corresponding mutant open the way to creating *MiMe* in this species.

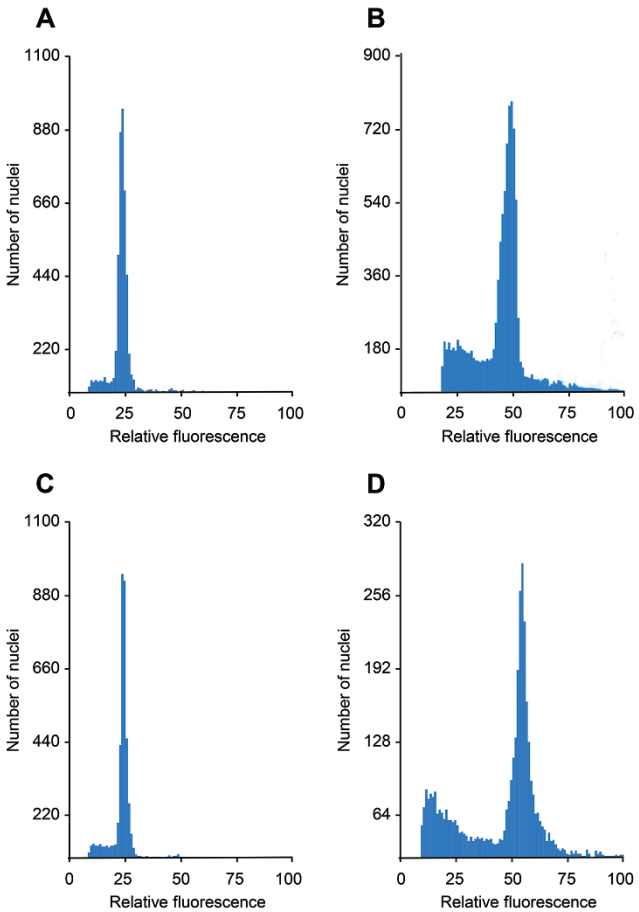

Figure 5 Ploidy of *Ososd1-1* pollen grains. The ploidy was determined using staining of isolated nuclei with propidium iodide followed by flow cytometry. **(A)** Wild-type Nipponbare pollen grains. A single peak is observed, corresponding to haploid nuclei. **(B)** Wild-type Nipponbare leaf. A single peak, corresponding to diploid nuclei. **(C)** *Ososd1*$^{+/+}$ pollen grains. A single peak is observed, corresponding to haploid nuclei. **(D)** *Ososd1*$^{-/-}$ pollen grains. A single peak is observed, corresponding to diploid nuclei.

Generating MiMe in rice

To generate the *MiMe* genotype, *Ososd1* mutation must be combined with a mutation that abolishes recombination, and *Osrec8* that modifies chromatid segregation. The *PAIR1* gene has been previously shown to be required for meiotic recombination initiation in rice [30]. One allele (Supplementary information, Figure S2) was selected in a Nipponbare background (AQUG12/*pair1-4*). As previously described for *pair1* alleles, plants homozygous for this insertion were fully sterile. In *pair1-4* meiocytes, 24 univalents were observed at metaphase I instead of 12 bivalents in wild type, followed by unbalanced distribution of univalents at anaphase I, suggesting that recombination was completely abolished by this allele (Figure 6A and 6B). *OsREC8* has also been functionally characterized [31]. *Osrec8* mutation leads to sister chromatid cohesion defect and chromosome frag-

mentation, similar to *Atrec8* [19, 32, 33]. We identified a novel T-DNA allele in the Hwayoung genetic background (Postech mutant line B01997/*Osrec8-3*) which caused full sterility (Supplementary information, Figure S2). Meiotic chromosome spreads from *Osrec8-3* meiocytes displayed entangled mass of DNA at metaphase I, followed by chromosome fragmentation and missegregation (Figure 6C and 6D), similar to effects of other *Osrec8* alleles. In summary, three single mutants *pair1-4*, *Osrec8-3* and *Ososd1-1* exhibited the same phenotypes as the three mutants used to build the *MiMe* genotype in *Arabidopsis*.

Combination of *pair1*, *Osrec8* and *Ososd1* mutations in rice was performed through sequential crossing of heterozygous plants (Figure 7A). In each generation, the segregation of mutant alleles followed the expected Mendelian segregation pattern. The *pair1 Osrec8* double mutant was sterile. In this double mutant, 24 univalents were observed, which were aligned on the metaphase plate and were segregated into two groups of 24 chromatids (Figure 6E and 6F). This shows that the *pair1* mutation abolishes chromosome fragmentation of *Osrec8*, indicating that *OsREC8* is required for efficient DNA double-strand break repair. It also shows mutating *OsREC8* in *pair1* modifies the mode of segregation of chromosomes: in *pair1* the two sister chromatids segregate as a single unit (monopolar orientation), leading to unbalanced segregation of univalents at anaphase I, while in *pair1 Osrec8* the pairs of chromatids segregate to opposite poles (bipolar orientation), leading to a balanced mitotic-like distribution of sister chromatids at anaphase I. Hence, like in *Arabidopsis* [19] and fission yeast [34], *OsREC8* is required for monopolar orientation of sister kinetochores at meiosis I in rice. In *pair1 Osrec8*, the free chromatids resulting from the mitotic-like segregation at meiosis I did not segregate properly at second division, producing unbalanced spores. The single mutants *pair1*, *Osrec8* and the double mutants *pair1 Osrec8* and *pair1 Ososd1* plants were fully sterile. In contrast, the *pair1 Osrec8 Ososd1* triple mutant showed higher fertility, similar to *Ososd1* single mutant (Figure 7B). Examination of the male meiotic chromosome behavior in *pair1 Osrec8 Ososd1* (Figure 8) revealed the presence of 24 univalents that aligned at metaphase I, and segregated into two groups of 24 chromatids to produce dyads of spores. This behavior resembles a mitotic division, suggesting that, like in *Arabidopsis,* the combination of *pair1*, *rec8* and *osd1* mutations turns meiosis into mitosis. We therefore term this genotype *OsMiMe*.

To gather further support that *OsMiMe* turns meiosis into mitosis, we asked if these plants produce diploid clonal gametes. Ploidy of seedling progeny from

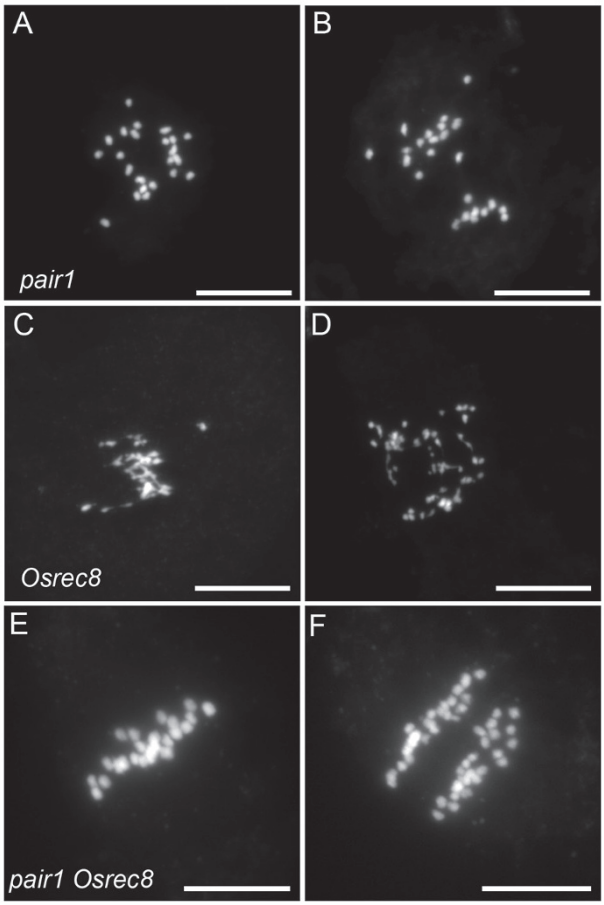

Figure 6 Male meiosis I in rice *pair1*, *Osrec8* and *pair1 Osrec8* mutants. **(A**-**B)** *pair1* (*n* = 154). **(A)** Metaphase I with 24 unaligned univalents. **(B)** Anaphase I with unbalanced segregation of univalents. **(C**, **D)** *Osrec8* (*n* = 222). **(C)** Metaphase I with abnormal chromosomes. **(D)** Anaphase I with chromosome fragmentation. **(E**, **F)** *pair1 Osrec8* (*n* = 154**)**. **(E)** Metaphase I with 24 aligned univalents. **(F)** Anaphase I with segregation of 24 pairs of chromatids. Scale bar = 10 μm.

self-pollination was estimated using flow cytometry, for both *Ososd1* and *OsMiMe* plants. All the progeny (from two and three mother plants, *n* = 50 each) were tetraploid, indicating that both *Ososd1* and *OsMiMe* diploid plants produce diploid male and female gametes. As the chromosome spreads suggested that gametes are derived from a first meiotic division in *Ososd1*, and from a mitotic-like division in *OsMiMe*, their genetic make-up should be different (Supplementary information, Figure S1). *OsOsd1* should produce recombined gametes, while *pair1 Osrec8 Ososd1* should produce clonal gametes. More specifically, segregation of a heterozygous marker (Aa) following meiosis I depends on its position along the chromosome [35]. A marker at the centromere would

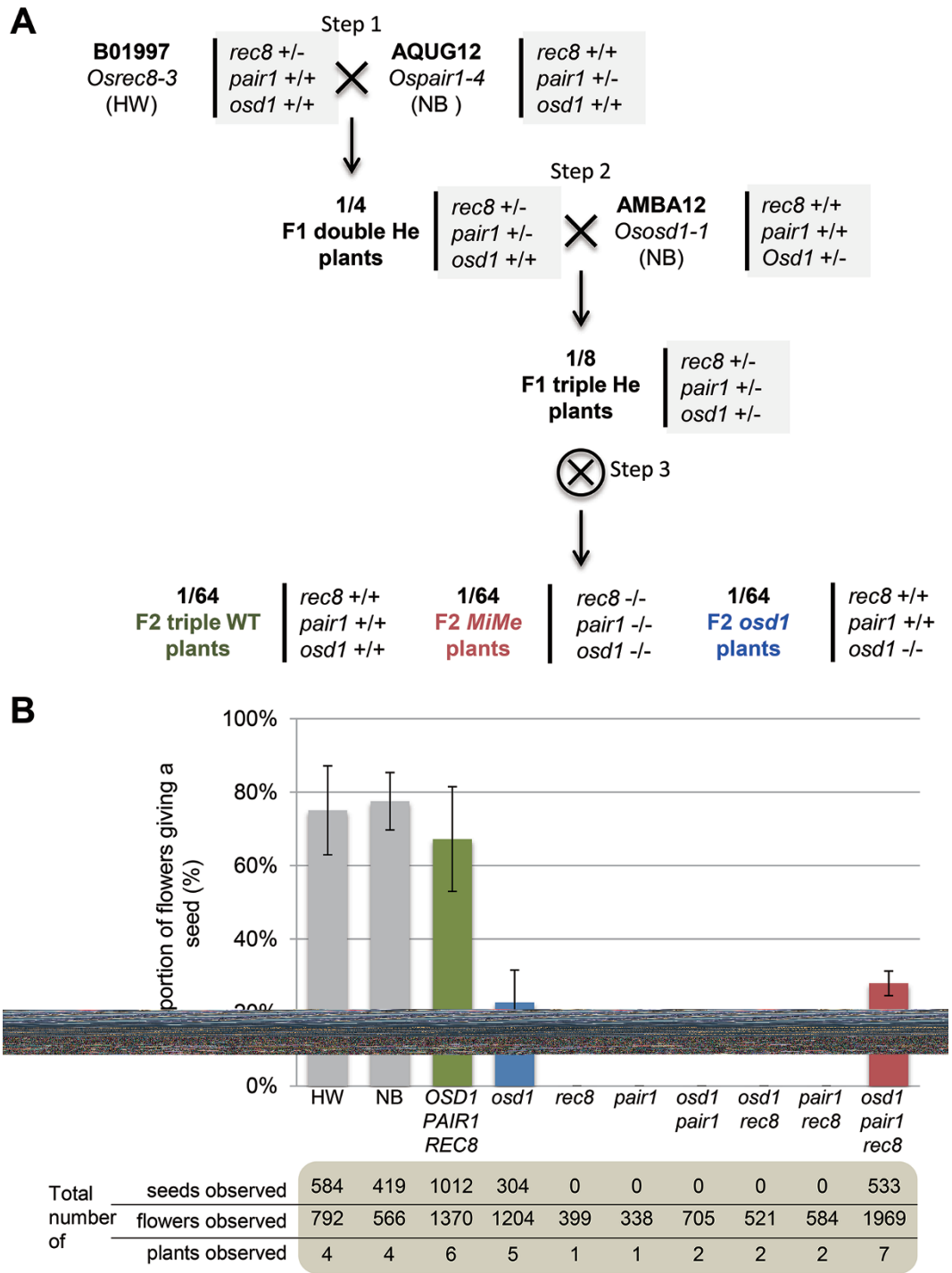

Figure 7 Creating *OsMiMe*. **(A)** Crossing scheme. Os*MiMe* mutant was created first by crossing B01997 plants heterozygous for the *Osrec8* mutation with AQUG12 plants heterozygous for the *pair1* mutation (step 1). 1/4 of F1 were double heterozygous plants and were in turn crossed with AMBA12 plants heterozygous for the *Ososd1* mutation to produce 1/8 of F1 triple heterozygous plants (*Osrec8*$^{+/-}$; *pair1*$^{+/-}$; *Ososd1*$^{+/-}$) (step 2). These plants were selfed (step 3) and the progeny were genotyped to identify 1/64 triple homozygous *OsMiMe* plants and relevant control segregants. HW, Hwayoung; NB, Nipponbare; He, heterozygous. **(B)** Fertility in *MiMe* and related mutants. The histogram represents the percentage of flowers giving a seed by self-fertilization. Numbers of observed flowers and seeds are indicated. Errors bars correspond to the SD for 4-7 plants.

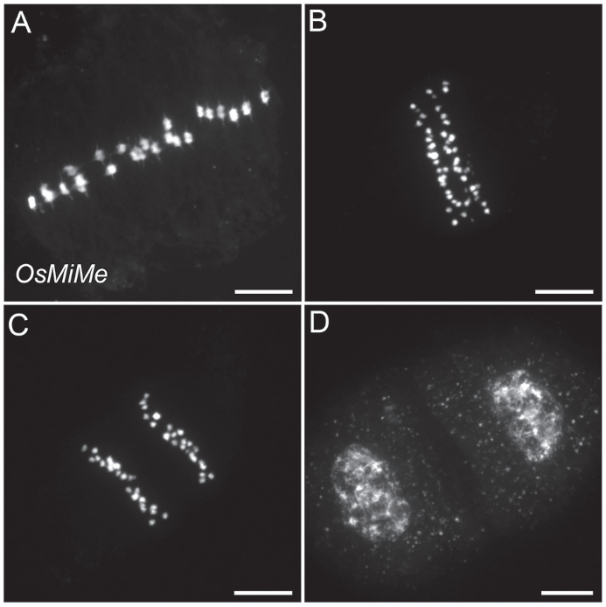

Figure 8 Male meiosis I in Os*MiMe*. **(A)** Metaphase I with 24 aligned univalents. **(B**, **C)** Anaphase I with segregation of 24 pairs of chromatids. **(D)** Telophase I. *n* = 43. Scale bar = 10 μm.

be homozygous in each diploid gamete (AA or aa), because of co-segregation of sister chromatids. This would render the tetraploid progeny after selfing having AAAA (¼ of plants), aaaa (¼ of plants), and AAaa (½ of plants). Markers distant from the centromere would segregate with different frequencies because of recombination [35]. The situation following a mitotic-like division is simpler: all heterozygosity would be maintained in each diploid gamete (Aa) because of separation of sister chromatids, and absence of recombination.

To analyze the segregation pattern in *Ososd1* and *pair1 Osrec8 Ososd1*, we took advantage of the fact that the genetic background of the segregating population is derived from a cross between two polymorphic strains Hwayoung (B01997/*Osrec8-3*) and Nipponbare (AQUG12/*pair1-4*), which was backcrossed to Nipponbare (AMBA12/*Ososd1-1*) (Figure 7A). We selected markers that were heterozygous in the *Ososd1* and *pair1 Osrec8 Ososd1* diploid plants. The genotypes of tetraploid progeny siblings of *Ososd1* and *pair1 Osrec8 Ososd1* diploid parents at diagnostic markers are shown in Figure 9. In *Ososd1* progeny (*n* = 77), the expected segregation was observed with the loss of heterozygosity at centromeric markers in ~50% of the plants. In sharp contrast, all the diagnostic markers heterozygous in the *OsMiMe* diploid parents were also found heterozygous in all the tetraploid progeny siblings (*n* = 73). This indicates that all *OsMiMe* gametes fully retained the parental plant genotype.

Discussion

Engineering apomixis in sexual crops would allow the self-propagation of F1 hybrids, which would have immense interest in agriculture by giving access to hybrid vigor in more species and to resource-poor farmers. Apomixis can be separated into two developmental components, the absence of meiosis or its modification into a mitosis-like division and the development of the embryo and its nourishing tissue from the female gametophyte without contribution of a male genome. The most efficient strategy to date to modify meiosis into mitosis has been obtained by combining mutations in three genes *SPO11-1*, *REC8* and *OSD1* (called the *MiMe* genotype) in the model species *Arabidopsis thaliana*. These three mutations affect, respectively, the three pillars of meiosis, homologous recombination, monopolar orientation of sister chromatids and the occurrence of a second division. Here we show that the mutation of *SPO11-1* can be efficiently replaced by mutation in other recombination initiation factors *PRD1*, *PRD2* or *PRD3/PAIR1* to create the *MiMe* phenotype. This suggests that mutation in any gene essential for double-strand break formation, such as *SPO11-2*, *DFO* or *MTOPVIB*, may be used instead of *SPO11-1* [22-26]. This observation may allow us to build *MiMe* in other species, in which mutant collection is limited or in which some genes could be present in more than one copy.

Although *MiMe* technology has been proven to be very efficient in the model species *Arabidopsis*, a major question has been about its transferability to crop species, especially the distant cereal species. *SPO11-1* and *REC8* are widely conserved among eukaryotes, both in sequence and in function, making the identification of their homologs in other plant species easily feasible. Other genes known to be essential for recombination initiation are also well conserved among plants and all these meiosis genes are typically represented by a single copy [28]. Indeed, mutating *REC8* or *PAIR1/PRD3* in rice recapitulates the phenotypes observed in *Arabidopsis*. In contrast, *OSD1* appears to be a plant-specific gene and exhibits a more complex phylogeny (Figure 2). There is one *OSD1* paralog in the *Arabidopsis* genome, *UVI4*, which has a distinct function in regulating somatic cell cycle [27]. *osd1 uvi4* double mutant confers very strong gametophyte and developmental defect, suggesting that these two genes have redundant functions in mitosis [36, 37]. Sequence analysis clearly distinguishes OSD1 from the UVI4 protein families in *Brassicaceae* species, but

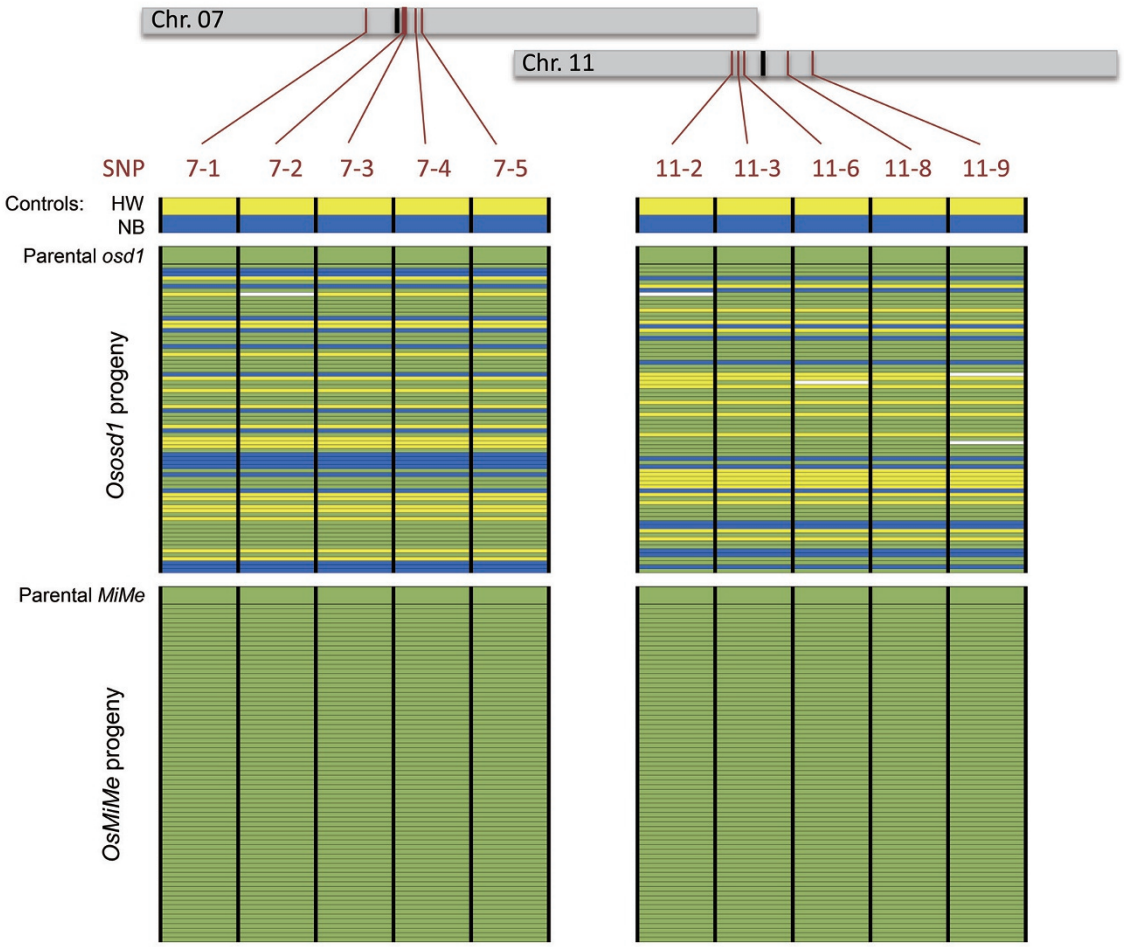

Figure 9 Genotypes of Os*Osd1* and Os*MiMe* diploid parentals and their respective tetraploid progeny siblings. Tetraploid offpring of *OsOsd1* and *OsMiMe* plants (refer to Figure 7 for scheme of creation of plant materials) were genotyped using 10 SNP markers residing on rice chromosomes 7 and 11. Positions of markers (brown) and centromeres (black) are indicated along the chromosomes. Each line represents a plant. For each marker, plants carrying only the Hwayoung allele are in yellow, plants carrying only the Nipponbare allele are in blue, while plants with both the Hwayoung and Nipponbare alleles appear in green. *Osd1* and *OsMiMe* parental plants (top line of upper and lower panels, respectively) are heterozygous at the 10 markers. While the offspring of *OsOsd1* exhibit segregation of parental alleles at the 10 markers, all the offspring of *OsMiMe* are heterozygous at the same 10 markers and exhibit the same genotype like the parental plants.

not in more distant flowering plants (Figure 2). In cereals, there are clearly two subgroups of genes, with one or two representatives of both groups in each species. The *Oryza sativa* genome contains two genes, one in each group, and we showed that the single mutation of Os02g37850 is sufficient to give rise to the meiotic defects observed in the *Arabidopsis osd1* mutant. We consequently called this gene *OsOSD1*. Meiosis in *Ososd1* skips the second division, producing diploid spores and gametes. These gametes are functional, leading to increased ploidy in the offspring by selfing. The existence and identification of the functional homolog of *OSD1* in rice suggests that it could be identified in other cereals. Synteny and protein sequence similarity indicate ortholog of *OSD1* as a single gene in *Hordeum vulgare*, *Brachypodium distachyon* and as a tandem duplication in *Zea mays*, *Sorghum bicolor* and *Setaria italica* [28]. This suggests that diploid gamete production through *osd1* mutations could be obtained in cereals, but may require the use of RNAi or genome editing technologies in the case of tandem duplications.

We identified three meiotic mutations that respectively abolish recombination (*pair1*), allow the separation of sister chromatids (*Osrec8*) and provoke the omission of second division (*Ososd1*). Next, we combined them to construct the *MiMe* genotype. In this triple mutant, meiosis is converted into a mitotic-like division with balanced

segregation of sister chromatids in a single division event. *OsMiMe* plants produce diploid gametes that are genetically identical to their parents. Our study thus establishes that the *MiMe* technology is transferable from the model plant *Arabidopsis* to the distant crop *Oryza sativa*, and suggests that it may be adopted in other important crops. The creation of the rice *OsMiMe* triple mutant was time-consuming and the approach remains so far restricted to genotypes/species with large associated mutant resources. However, recent breakthrough in genome editing by CRISPR/Cas9 may remove many obstacles [38], making apomeiotic conversion a feasible reality in many crops.

Rice *OsMiMe* plants produce functional clonal embryos. However, fertilization still occurs normally, leading to the doubling of ploidy in the next generation. The next challenge is to trigger the development of a *MiMe* gamete into an embryo and then a plant without the contribution of another gamete. One approach is to use genome elimination, where the chromosome set from one parent is removed after fertilization in the zygote. *MiMe* combined with genome elimination indeed leads to clonal offspring, as previously demonstrated in *Arabidopsis* [13]. However, the CENH3 manipulation used in *Arabidopsis* to provoke genome elimination has not been transferred in other species to date [20, 39-41], except in maize but with a lower frequency [42]. The frequency of genome elimination can be genetically controlled in cereals, notably in maize [43-45]. The *gynogenesis inducer 1* (*ggi1*) locus has been shown to induce genome elimination and is widely used in maize breeding to produce haploids and the identification of the underlying gene(s) will open exciting new avenues to clonal reproduction [46]. Alternatively, parthenogenic development of embryo could be induced, and recent progress has been made in the understanding of its genetic control [47, 48].

Materials and Methods

Phylogeny

Protein sequences from the OSD1/UVI4 family were collected using PLAZZA DICOTS3.0 and PLAZZA Monocots 3.0 (http://bioinformatics.psb.ugent.be/plaza/) [49] and correspond to family HOM03M004665, HOM03M067707 and HOM03D003883. Some species were removed from the analysis for simplicity. BD5G13210 sequence was replaced by Bradi5g13212.1 (Phytozome 10.3.). Three proteins (SI005G06210, ZM10G18380 and GM11G21435) were excluded because, though they share sequence similarity with the OSD1/UVI4 proteins, they lack most of the conserved domains. Multi-protein alignment (Muscle v3.8) followed by phylogeny inference (PHYLIP Neighbor-Joining/UPGMA method version 3.696) was performed using the MPI toolkit with default parameters [50].

Genetic material

The *Arabidopsis* mutants used were *prd1-2*, *prd2-1*, *prd3-3* [22, 23], *osd1-1* and *atrec8-3* [16]. Rice (*Oryza sativa* L.) mutants used in this study were the following: AMBA12 T-DNA insertion line and two Tos17 insertion mutant AMQF10 and ALJA10 lines for OsOSD1 (Os02g37850); AQUG12 Tos17 insertion line for PAIR1 (Os03g01590); B01997 T-DNA insertion line for OsREC8 (Os05g50410). All insertion lines were generated in the Nipponbare background and are from the Oryza Tag Line insertion line library [51, 52] except B01997, which has a Hwayoung background and is from the POSTECH Rice Insertion Database [53]. Position of the inserts in the respective genes is shown in Supplementary information, Figure S2. The exact junction of the T-DNA insert with the rice genome in the mutants was ascertained by Sanger sequencing.

Genotyping of T-DNA and Tos17 inserts

Genotyping primers are listed in Supplementary information, Table S1**.** Primers were designed using the "Genotyping Primer Designer" tool of OryGenesDB (http://www.orygenesdb.cirad.fr) [54, 55]. In these standard conditions, the expected PCR product is ~800 bp for a mutant allele and ~1 000 bp for a wild-type allele. PCR was performed following the protocol described in [56].

SNP genotyping

Single-nucleotide polymorphism genotyping was performed using Kompetitive Allele Specific PCR (KASP) following the LGC group recommendations for the use of KASP technology on Roche LC480.

Cytological analyses

Arabidopsis chromosome spreads and observation were carried out using the technique described in [18].

Conditions of growth and crossing of the plants

Rice plants were grown under containment greenhouse condition (temperature of 28 °C day and 24 °C night, 60% hygrometry) under natural light that could be assisted by artificial sodium light (light intensity of 700 µmol/m^2/s). Crossing was accomplished through manual castration of florets and pollination according to the standard procedures, followed by bagging to avoid pollen contamination.

Embryo rescue

Five days after pollination, fertilized flowers were harvested, their lemma and palea removed under a stereo microscope and soaked in 70% ethanol for 5 min. The caryopses were then washed thrice in sterile distilled water. Embryos were isolated following two distinct procedures depending on the presence or absence of endosperm [42, 43]. Immature embryos were plated on MS basal medium [44] supplemented with 50 g/l sucrose and 6 g/l phytagel. Following a 10-day growth period in the dark at 26 °C, the seedlings were transferred to a fresh MS solid medium (2.6 g/l of phytagel; 30 g/l of sucrose) and in a growth chamber (photoperiod 16:8), 28 °C and 60% humidity for 3 weeks.

Flow cytometry determination of DNA content in pollen and leaf cell nuclei

Ploidy was determined by estimating nuclear DNA content

by flow cytometry. Pollen grains from flowers at anthesis were collected by introducing the panicle into a 50 ml tube and gently shaking allowing the shedding of pollen from anthers. One milliliter of LB01 buffer was added to humidify the pollen followed by a mild centrifugation (10 min at 150× *g*) allowing collection of the pollen pellet. The supernatant was discarded and the pellet was re-suspended with 1 ml of a fresh LB01. The nucleus extraction from pollen grains was performed according to Kron *et al*. [57] bursting method. Due to the size of rice pollen grains and nuclei, we used Partec CellTrics at 100 µm for the first filtration and 30 µm for the second one. For leaf nucleus extraction, ~0.5 cm^2 of leaf blade tissue was chopped in 250 µl of LB01 extraction buffer. The suspension was filtered (30 µm Partec CellTrics filters) to eliminate cell debris. After filtration, 700 µl of LB01 buffer was added. After DNA nucleus extraction from pollen or leaf tissue, DNA was stained by addition of propidium iodide (40 µl of PI at 0.2 mg/ml). The samples were analyzed with UV excitation in a Partec flow cytometer (PA-I; Partec, Munster, Germany).

Acknowledgments

This work has been funded by the ANR Emergence DiplOSD (ANR-09-EBIO-011) to RM and EG. Part of this work has been supported by the RicE FUnctional GEnomics (REFUGE) platform funded by Agropolis Fondation. We thank Eve Lorenzini, Christian Chaine and Rémy Michel for providing technical assistance, notably in greenhouse. We thank the guidance of Dr Frédéric Bakry, Cirad, for flow cytometric analyses. We thank Marta Cifuentes, Eric Jenczewski, Wayne Crismani, Rajeev Kumar and Anne Diévart for critical reading of the manuscript. The IJPB benefits from the support of the Labex Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS).

Author Contributions

EG and RM conceived the project. DM, EG and RM designed experiments. DM, SJ, MR, LC, AV, PM, LP, GD and BD performed experiments and analysed the data. DM, EG and RM wrote the paper with the input from other authors.

Competing Financial Interests

The authors declare no competing financial interests

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)