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# Supporting Online Material for

## Synthetic Clonal Reproduction Through Seeds

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## Abstract

Cloning through seeds has potential revolutionary applications in agriculture, because it would allow vigorous hybrids to be propagated indefinitely. However, asexual seed formation or apomixis, avoiding meiosis and fertilization, is not found in the major food crops. To develop de novo synthesis of apomixis, we crossed *Arabidopsis MiMe* and *dyad* mutants that produce diploid clonal gametes to a strain whose chromosomes are engineered to be eliminated after fertilization. Up to 34% of the progeny were clones of their parent, demonstrating the conversion of clonal female or male gametes into seeds. We also show that first generation cloned plants can be cloned again. Clonal reproduction through seeds can therefore be achieved in a sexual plant by manipulating two to four conserved genes.

# Supporting Online Material

## Materials and methods

### Plant material and growth conditions

Plants were grown in artificial soil mix at 20ºC under fluorescent lighting. Wild type and mutant strains of *Arabidopsis* were obtained from ABRC, Ohio or NASC, UK. *dyad* was crossed to the No-0 strain to generate populations that were heterozygous for markers across the genome. *MiMe* plants were a mixture of Col-0 from *Atspo11-1-3/Atrec8-3* and No-0 from *osd1-1* (*S1*). The *GEM* plants used in this study are $F_1$ progeny obtained by crossing *cenh3-1/cenh3-1 GFP-tailswap/GFP-tailswap* (female) to *cenh3-1/cenh3-1 GFP-CENH3/GFP-CENH3 (male).*

### Genotyping and microsatellite marker analysis

Primers for *osd1-1*, *Atspo11-1-3* and *Atrec8-3* (*MiMe*) genotyping are described (*S1*). Microsatellite markers (Table S3) were analyzed as described (*S1-S3*). Primer sequences were obtained from TAIR (www.arabidopsis.org) or from the MSAT database (INRA).

*cenh3-1:* a point mutation G161A in the *CENH3* gene (also known as *HTR12*) detected with dCAPS primers (dCAPs restriction polymorphism with EcoRV, the wild-type allele cuts):

Primer 1: GGTGCGATTTCTCCAGCAGTAAAAATC

Primer 2: CTGAGAAGATGAAGCACCGGCGATAT

Detection of *GFP-tailswap* insertion on chromosome 1:

Primer 1 for wild type and T-DNA: CACATACTCGCTACTGGTCAGAGAATC

Primer 2 for wild type only: CTGAAGCTGAACCTTCGTCTCG

Primer 3 for the T-DNA: AATCCAGATCCCCCGAATTA

Primers for detection of *GFP-CENH3*:

CAGCAGAACACCCCCATC (in *GFP*)

CTGAGAAGATGAAGCACCGGCGATAT (in *CENH3*)

**Ploidy analysis**

*MiMe* and *osd1* offspring ploidy analyses were performed by flow cytometry and systemically confirmed by chromosome spreads as described (Figure S4 A-C) (*S1, S2*). For *dyad* offspring, ploidy analysis was by flow cytometry and randomly selected diploid eliminants (n=5) were further confirmed by FISH analysis using a centromere repeat probe to count chromosomes *(S4*) and all were found to be diploids (Figure S4 D). Isolation of nuclei for flow cytometry was performed as described (*S5*). Flow cytometry analysis was carried out using an internal diploid and tetraploid control to unambiguously identify diploid plants.

In elimination crosses to the wild type tetraploid line (C24 background), triploids were identified as late flowering (due to combination of the Col-0 *FRIGIDA* and C24 *FLOWERING LOCUS C* alleles). The aneuploid plants show distinct morphological phenotypes such as altered vegetative growth, variation in rosette leaf morphology (size and shape), a range of leaf color (pale yellow to dark green) and thus can be easily distinguished from normal diploid wild-type plants (Fig. S5). Further, aneuploid plants show varied flowering time and mostly have reduced fertility and seed set (*S6, S7*). Putative diploids were genotyped for at least one marker per chromosome (Chr 1: F5I1, CIW12; Chr 2: MSAT2.11; Chr 3: MSAT3.19, CIW11; Chr 4: nga8; Chr 5: CTR1.2, nga106). Eliminants were identified as having only C24 alleles, in addition to lacking GFP fluorescence at the centromeres which is present in the *GEM* line. Random diploid plants (n=8) were further confirmed by karyotyping in meiotic chromosome spreads and all were found to be diploids (Fig.S5).

## Supporting text

### Development of an efficient elimination line for diploid gametes

The *GFP-tailswap* line (*cenh3-1* mutant plants rescued by a *GFP–tailswap* transgene) is an efficient haploid inducer (*S8*), but is difficult to cross as the pollen donor because it is mostly male sterile. Further, *GFP-tailswap* plants give an extremely low frequency of viable seeds (2%) when crossed as female to a tetraploid male that produces diploid gametes (*S8*). *GFP-CENH3* (*cenh3-1* mutant plants rescued by a *GFP–CENH3* transgene) is a weaker haploid inducer but is much more fertile (S*8*). We hypothesized that co-expression of *GFP-CENH3* and *GFP-tailswap* in *cenh3-1* plants would produce more viable pollen and give better seed set than *GFP-tailswap*, yet still induce genome elimination when these plants were crossed to wild-type tetraploid plants. Indeed, *cenh3-1* plants carrying both *GFP-CENH3* and *GFP-tailswap* transgenes produced ample pollen for crosses, although pollen viability was still lower than wild-type (Fig. S1). When these co-expressing plants were crossed as female or male to tetraploid wild-type, seed viability was much higher (40% and 80% respectively) compared to the *GFP-tailswap* cross and their chromosomes were eliminated in a subset of F1 progeny (Table S1). We named the line *GEM* (Genome Elimination caused by a Mix of CENH3 variants). In summary, *GEM* is fertile as either male or female, and shows efficient genome elimination when crossed to a parent that makes diploid gametes.

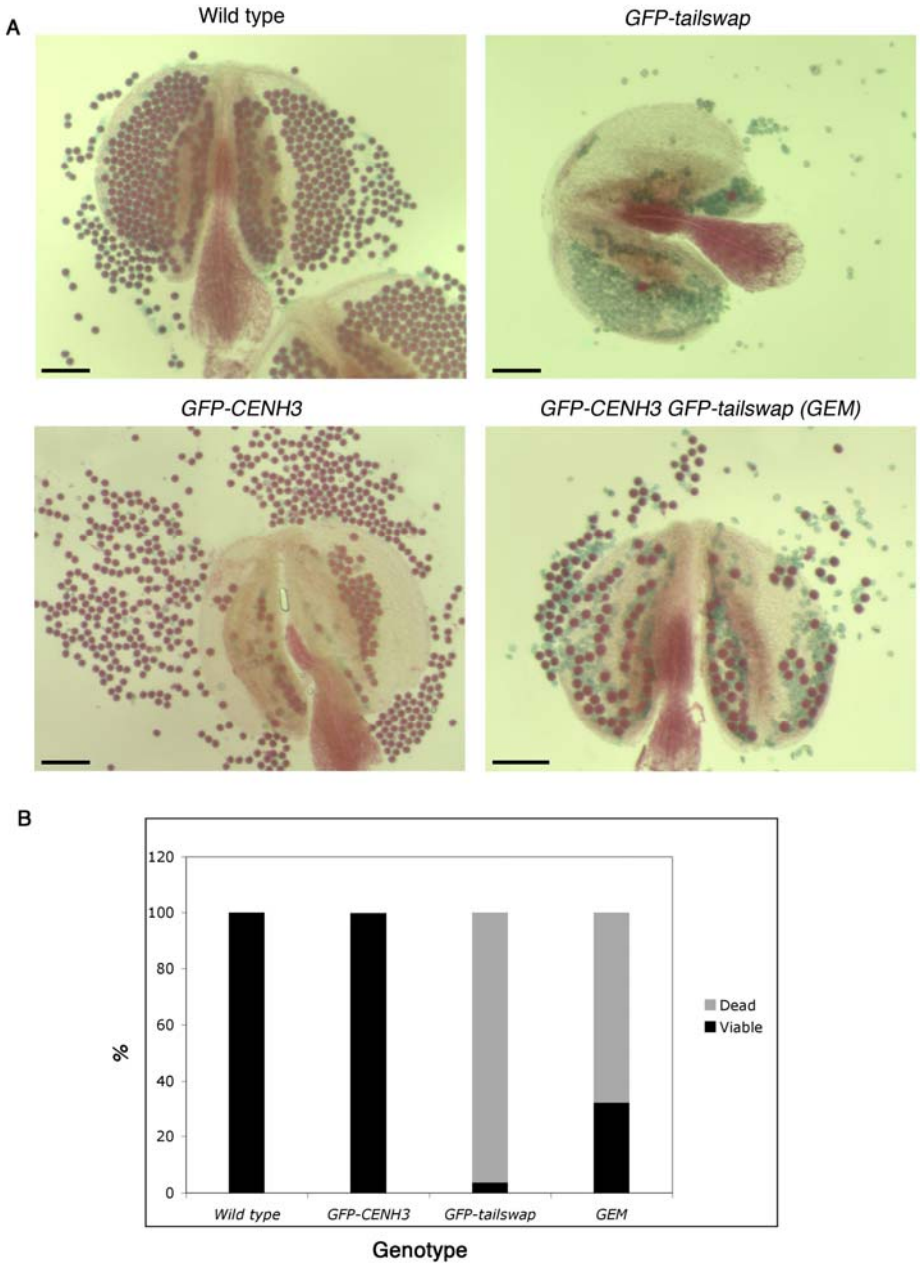
### Crosses between *osd1* and *GEM* lead to diploid uniparental but recombined progenies.

We previously showed that *osd1* diploid mutants produce diploid male and female gametes because of an absence of the second division of meiosis (*S1*). We reasoned that crossing *osd1* to *GEM* should give rise to diploid progeny that originated only from the diploid *osd1* parent because of elimination of the *GEM* parental genome. We tested our assumption by taking advantage of the three different genetic backgrounds of the *osd1-1* (No-0) and *osd1-2* mutants (Ler) and *GEM* (Col-0). We crossed *osd1-1/osd1-2* plants that were heterozygous for polymorphisms between No-0 and Ler, to *GEM* and followed parental origin in the progeny using trimorphic markers.

Crossing *osd1-1/osd1-2* as female with *GEM* as male resulted in 29 viable seeds per fruit, 26% of them being diploid (Table S1). Among these diploid progeny, half of them (24/50) were from sexual origin, carrying alleles of both parents (Fig. S2A). These plants likely originated from fertilization of the ~15% of haploid female gametes produced by *osd1* mutants (*S1*) with haploid pollen made by GEM and no subsequent chromosome elimination. The other half of the diploid progeny (26/50) carried only maternal alleles at every locus tested (Fig. S2A). These diploid eliminant plants also exhibited the *osd1* phenotype like their mother, having wild type somatic development and producing a dyad of spores instead of a tetrad after meiosis. Moreover, the genotype of these plants perfectly reflected the genotype of the *osd1-1/osd1-2* gametes. Indeed, because *osd1* mutant gametes are produced following a single first division of meiosis, heterozygosity at centromeres is lost in the diploid gametes because of co-segregation of sister chromatid centromeres during this division. Because of recombination that occurs during the first division, any loci which are not linked to a centromere segregate in the *osd1* diploid gametes (*S1*). The genotypes of the diploid eliminant plants we obtained showed exactly this pattern (Fig. S2A., the μ marker is a centromeric locus), confirming that their genome originated exclusively from *osd1* diploid maternal gametes and that the plants are thus gynogenetic.

We next tested the possibility of androgenesis by crossing *GEM* as female with *osd1-1/osd1-2* as male. This resulted in 14 seeds per fruit (Table S1), of which 25% germinated (3-4 viable seeds per fruit). Of the germinated seeds, 20% were diploid suggestive of androgenesis because *osd1* produces only 2n pollen grains (*S1*). All these 2n plants carried exclusively paternal alleles (Fig. S2B) and exhibited the *osd1* phenotype like their father. These diploid plants were thus of paternal origin. As for the previous cross, their genotype reflected the genotype of *osd1* gametes, being recombined and having lost paternal heterozygosity in the vicinity of centromeres (Fig. S2B). These progeny are thus androgenetic having used *GEM* as a surrogate mother.

**Figure S1. *GEM* produces higher frequency of viable pollen than *GFP-tailswap*.**




A. Vital staining of pollen grains by Alexander staining in the genotypes indicated. Viable pollen stains pink/red whereas dead/inviable pollen stains green. Scale bar =125µm

B. Percentage of viable (black) and dead (grey) pollen from genotypes indicated.

**Figure S2. Genotype analysis of *osd1* x *GEM* and *GEM* x *osd1* offspring.**

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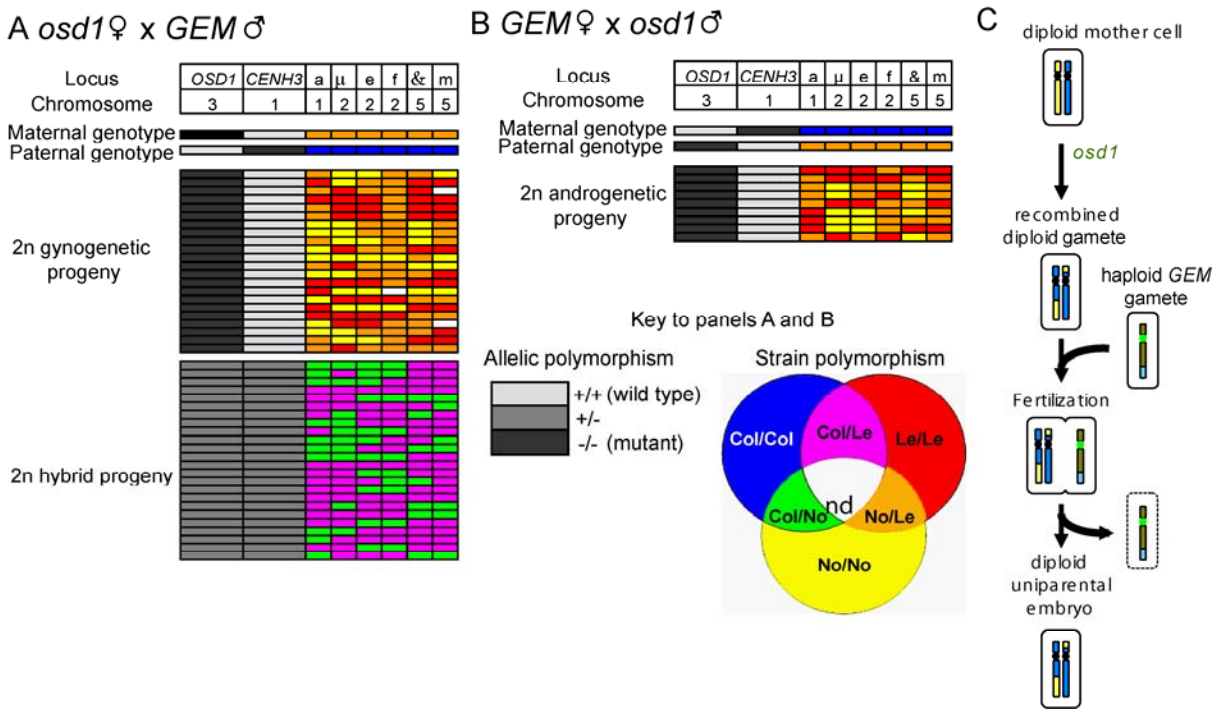
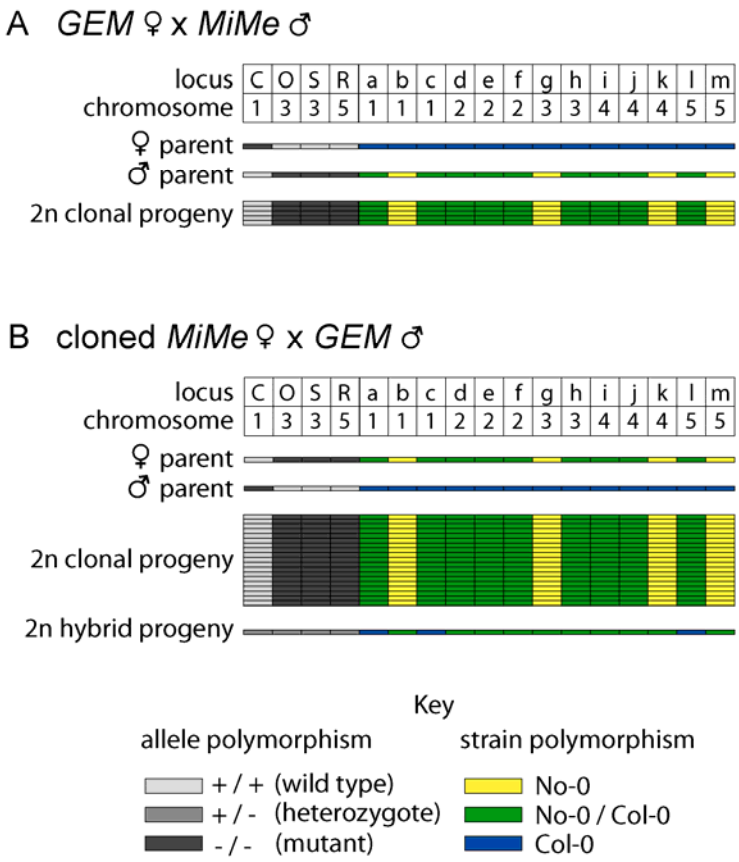


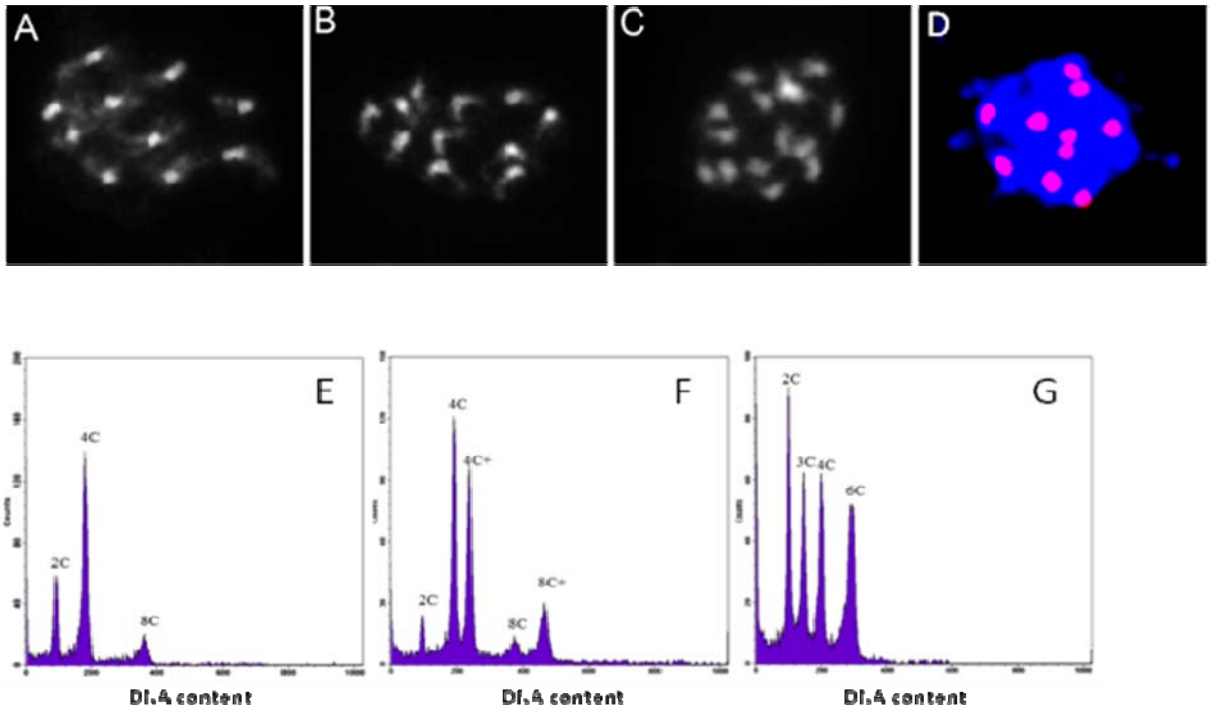

Diploid offspring of the crosses, identified by flow cytometry and confirmed by mitotic chromosome spreads, were genotyped for parental mutations and several trimorphic molecular markers (see Table S3). Each line represents one plant. For each mutation, the wild type genotype is represented in light grey, the homozygote mutant genotype in dark grey and the heterozygote in medium grey. For each marker, the genotype is encoded according to the color rosette. Markers in white were not determined. For each cross, the two first lines represent the parental genotype. (A) *osd1*♀ x *GEM*♂. Among the diploid plants, half had a genotype of maternal origin (recombined), lacking paternal contribution (gynogenetic progeny) and the other half had a hybrid genotype. (B) *GEM*♀x *osd1*♂. Among the diploid plants, all had a genotype of paternal origin (recombined), lacking maternal contribution (androgenetic progeny). (C) Schematic representation of the mechanisms of production of diploid uniparental recombined progeny.

**Figure S3. Genotype analysis of *GEM* x *MiMe* and cloned *MiMe* x *GEM* offspring.**



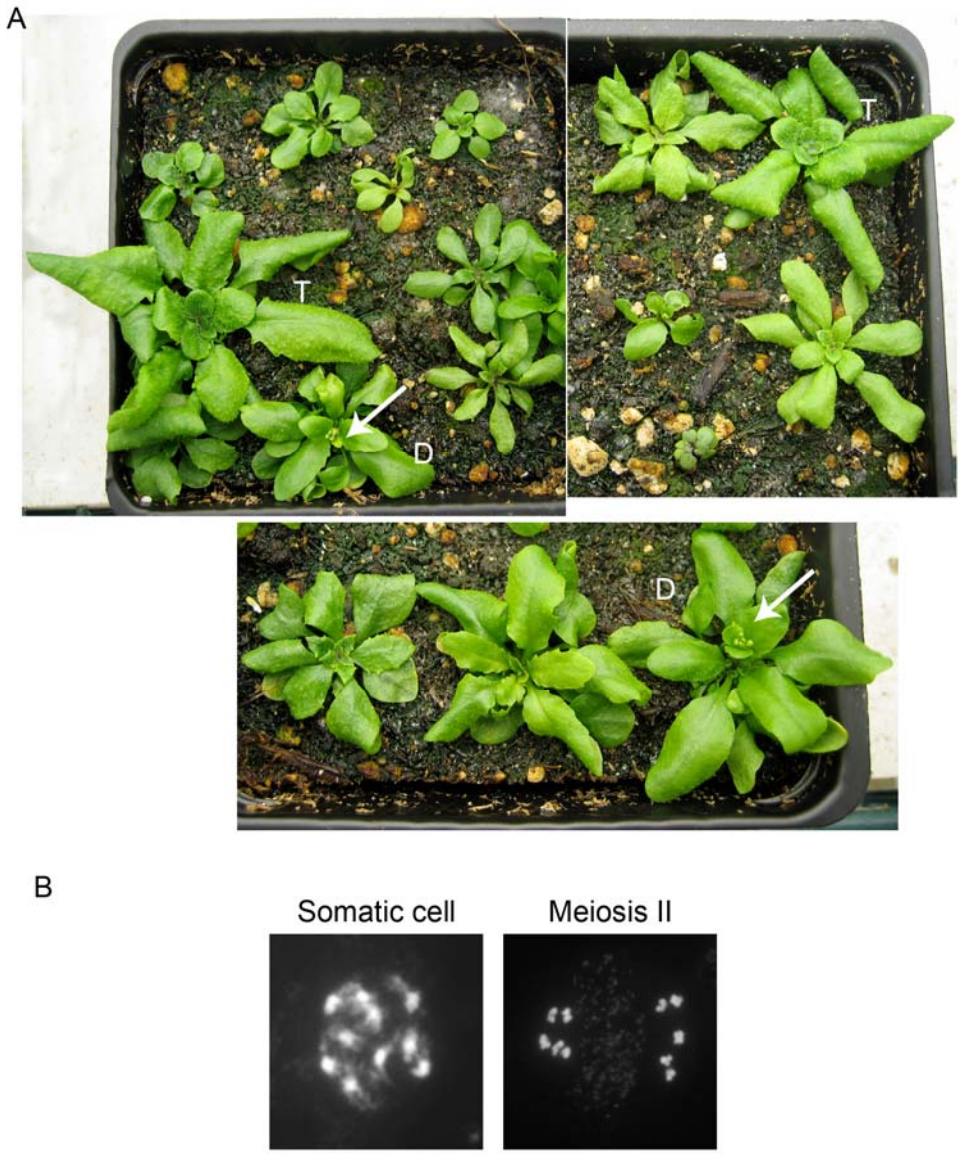

Parents and diploid progeny were genotyped for polymorphic loci (Table S3). Each row represents one plant and each column is a locus.

**Figure S4. Flow cytometry and chromosome spreads of clonal, aneuploid and sexual progeny of *Mime* x *GEM* and *dyad* x *GEM* crosses.**




(A-C) *MiMe* x *GEM* progeny (A) diploid eliminant, 10 chromosomes. (B) aneuploid with 11 chromosomes (C) Triploid, 15 chromosomes. (D) FISH of *dyad* x *GEM* diploid eliminant, 10 chromosomes. (E-G) Flow cytometry of *dyad* x *GEM* progeny with diploid as internal control. (E) diploid. (F) aneuploid -- 4C+, 8C+ peaks. (G) triploid. Endoreduplication in *Arabidopsis thaliana* leaves means that peaks at 2x and 4x the base DNA content may be observed.

**Figure S5. Phenotypes of F1 progeny derived from a cross between *GEM (*2n) Col-0 strain x tetraploid wild type (4n) C24 strain.**




A. A range of morphological phenotypes was seen in aneuploids. D - Diploid, T- Triploid. Unlabelled plants are aneuploids. Triploids are more vigorous and robust than diploid and aneuploid siblings. Diploid clonal progeny originating exclusively from C24 parent diploid gametes are early flowering (arrows) when compared to all triploid/ most of aneuploid hybrid siblings that are late flowering (see Materials and Methods). B. Somatic and meiosis II (metaphase II) cell from a diploid showing 2n=10 karyotype.

**Table S1: Analysis of crosses between *GEM* and 4n wild type or *osd1*.**

| cross (♀ x ♂) | Seeds per silique | Germination rate | Total progenies analyzed | Triploid | Aneuploid | diploid | |
|---|---|---|---|---|---|---|---|
| | | | | | | Hybrid* | Uniparental* |
| Wild type 4n *x GEM* | 35 | 81% | 85 | 62% (53) | 32% (27) | N/A | 6% (5) |
| *GEM x* Wild type 4n | 20 | 40% | 84 | 14% (12) | 68% (57) | N/A | 18% (15) |
| *osd1* x *GEM* | 31 | 93% | 194 | 31% (60) | 43% (85) | 12% (24) | 13% (25) |
| *GEM* x *osd1* | 14 | 25% | 49 | 24% (12) | 55% (27) | 0% | 20% (10) |

The given percentages stand for the frequencies of aneuploid, triploid, diploid hybrid and uniparental diploid among the total progenies analyzed. The number of corresponding plants is indicated in brackets. * deduced from Figure S2. Tetraploid wild type was in the C24 accession. Seed set in diploid wild type was 50 seeds per silique.

**Table S2: Analysis of crosses between *GEM* and *MiMe* or *dyad*.**

| cross (♀ x ♂) | Seeds per silique | Germination rate | Total progenies analyzed | Triploid | Aneuploid | diploid | |
|---|---|---|---|---|---|---|---|
| | | | | | | Hybrid* | Clones* |
| *MiMe x GEM* | 15 | 92% | 155 | 13% (20) | 53% (82) | 0.6% (1) | 34% (52) |
| *dyad* x *GEM* | 0.9 | 73% | 220 | 18% (40) | 55% (121) | 14% (30) | 13% (29) |
| *GEM x MiMe* | 23 | 0.5% | 12 | 25% (3) | 33% (4) | 0% | 42% (5) |
| cloned *MiMe* x *GEM* | 14 | 91% | 79 | 20% (16) | 54% (43) | 1.3% (1) | 24% (19) |

The given percentages stand for the frequencies of aneuploid, triploid, diploid hybrid and clones among the total progenies analyzed. The number of corresponding plants is indicated in brackets. Progenies analyzed were from one mother plant for all *Mime* crosses and three mother plants in the case of the *dyad X GEM* cross. From individual *dyad* mother plants, the frequency of clonal seeds was 10% (10/96 progeny), 12% (4/34 progeny), and 17% (15/90 progeny) respectively. In the table, this is reported as a pooled value of 13% (29/220). * deduced from figure 1 data.

Triploid hybrid (3n=15) plants originate by the fertilization of a diploid gamete (2n=10) from either *MiMe* or *dyad* parent with that of a haploid gamete (n=5) from the *GEM* parent, without genome elimination.

Aneuploid plants can arise in two possible ways:

1. When a diploid gamete (from *MiMe* and *dyad*) is fertilized by a haploid (n=5)/aneuploid gamete (n >5) from *GEM* parent followed by incomplete genome elimination during zygotic mitosis.
2. When a haploid gamete (from *MiMe* and *dyad*) is fertilized by an aneuploid gamete (n >5) from *GEM*. *GEM* may produce viable aneuploid gametes at a varying frequency (unpublished observations).

Diploid hybrid plants orginate from fusion of haploid gametes from *MiMe* or *dyad* with haploid gametes from the *GEM* parent.

Diploid clones arise by the fertilization of diploid gametes from *MiMe* or *dyad* with haploid/aneuploid gametes from *GEM* followed by complete elimination of all *GEM* parent chromosomes in the zygote during mitotic divisions.

**Table S3: List of markers used in this study.**

| | | | |
|---|---|---|---|
| C | *CENH3* | O | *OSD1* |
| S | *SPO11* | R | *REC8* |
| a | f5i14 | n | NGA63 |
| b | msat1.13 | o | NGA280 |
| c | msat1.1 | p | NGA1145 |
| d | msat2.17 | q | NGA168 |
| e | msat2.21 | r | NGA162 |
| f | msat2.9 | s | GAPAB |
| g | msat3.32 | t | NGA6 |
| h | msat3.07194 | u | NGA1107 |
| i | msat4,02575 | v | NGA225 |
| j | mast4.35 | w | CA72 |
| k | msat4.18 | x | NGA139 |
| l | ath5S0262 | y | SO262 |
| m | nga76 | & | NGA151 |
| | | µ | msat2.18 |

## Supporting references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