Engineering apomixis in tomato   
for clonal seed development

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Data availability

All code used for all analyses and plots is publicly available on GitHub at: <https://github.com/MaartenWessel/Apomixis>

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# Abstract

Apomixis or asexual seed formation leads to clonal offspring. Introducing this trait into crops like *Solanum lycopersicum* (tomato) would provide new alternatives to conventional plant breeding. Apomixis is rare but naturally present in nature and has successfully been engineered in *Oryza sativa* (rice) and *Arabidopsis thaliana*. Apomixis consists of three components: apomeiosis (bypassing meiosis), parthenogenesis (embryo development without fertilization of the egg cell), and autonomous endosperm development (endosperm formation without fertilization of the central cell). This study focusses on engineering apomixis in Micro-Tom by evaluating the effects of genetic constructs targeting the skipping of meiosis II and the induction of parthenogenesis. Additionally, two transformations were performed to explore an alternative to skipping meiosis II and for identification of parthenogenesis. Here we show that introducing a *SlTDM1-P19L* mutation results in partial skipping of meiosis II in several tomato lines, indicated by the formation of larger, likely diploid pollen. This study demonstrates that mutating the phosphorylation site in *SlTDM1* is a viable way of inducing the skipping of meiosis II, although it is accompanied with a reduction in fertility. Furthermore, egg-cell specific expression of *ToPAR* leads to reduction in fertility. This finding advances our understanding of how meiotic progression in tomatoes can be genetically altered. Establishing apomeiosis and parthenogenesis in tomato lays the groundwork for clonal seed development in dicot crops. This research represents a step towards engineering apomixis into crops.

# Keywords

Apomixis, Apomeiosis, Parthenogenesis, Solanum lycopersicum, TDM1, PAR, Meiosis II, skipping meiosis II.

# Introduction

Traditional tomato breeding relies on repeated cross-pollination between homozygous parental lines to generate F₁ hybrids with improved growth and higher yields (Bai & Lindhout, 2007; Labroo et al., 2021). While effective, this process is labour-intensive, time-consuming, and must be repeated continuously. An alternative approach is the engineering of apomixis. Apomixis is a form of asexual seed formation that produces clonal seed genetically identical to the mother plant (Underwood & Mercier, 2022). By enabling seed-based propagation of heterozygous plants, apomixis could eliminate the need for continuous hybrid plant production. Although synthetic apomixis has been successfully demonstrated in rice (Khanday et al., 2019; Song et al., 2024; Vernet et al., 2022), its implementation in other crops remains challenging. Establishing apomixis in tomato could streamline breeding and fix desirable traits of good lines, reducing the dependency on continuous selective breeding.

Apomixis occurs in approximately 0.1% of all flowering plants (Hojsgaard et al., 2014). Although relatively rare, it is found across diverse species (Xu et al., 2022), with dandelions being a well-known example. The development of apomixis can be divided into three components. First, apomeiosis, the formation of diploid non-recombinant gametes through the bypassing of meiosis. Second, parthenogenesis, the development of an embryo without fertilization (see Figure 1). Third, autonomous endosperm development, the formation of endosperm without fertilization (Underwood et al., 2022). This research focuses on engineering apomeiosis and parthenogenesis.

A diagram of a diagram of a cell

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**Figure 1. Apomixis.** During wild-type sexual reproduction, the mother cell undergoes meiosis, producing four recombinant haploid gametes. Approximately one-quarter of these female gametes develop into egg cells, which are then fertilized by sperm cells to form hybrid embryos. In apomictic reproduction, the mother cell undergoes apomeiosis, generating two clonal diploid gametes. Fertilization is bypassed during parthenogenesis, resulting in the formation of a clonal embryo.

## Apomeiosis

Apomeiosis, which literally means “away from meiosis”, is the first step towards engineering apomixis. Under normal meiosis, a diploid cell undergoes two successive meiotic divisions to produce four haploid, recombinant cells. In contrast, apomeiosis produces two diploid, nonrecombinant cells. This process more closely resembles mitosis, as the resulting cells are genetically exact copies of the mother cell.

A synthetic form of apomeiosis was developed in *Arabidopsis thaliana,* by combining multiple mutations effectively modifying meiosis into a mitosis-like process (d’Erfurth et al., 2009)*.* Three stages of meiosis were genetically modified (see Figure 2). The first mutation, *SPORULATION11-1* (*spo11-1)*, inhibits meiotic recombination and chromosome pairing. The second mutation, *MEIOTIC RECOMBINATION PROTEIN8* (*rec8)*, results in the segregation of sister chromatids during the first meiotic division. The third mutation, *OMISION OF SECOND DIVISION1* (*osd-1)*, skips the second meiotic (Meiosis II) division. Together, these mutations form the “mitosis instead of meiosis” system (*MiMe*).

A diagram of a cell division

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**Figure 2. Comparison of wild-type meiosis and MiMe (mitosis instead of meiosis)** (Underwood & Mercier, 2022)**.** Mutations in spo11-1 (or pair1), rec8 and osd1 (or tam, tdm1-p17), convert meiosis into a mitosis-like division, by preventing recombination, altering chromatid segregation, and skipping meiosis II. These modifications result in the formation of two clonal diploid spores instead of four recombinant haploid spores.

Efforts to introduce *MiMe* in tomatoes have been made (*Wang et al - 2024 - Harnessing Clonal Gametes in Hybrid Crops to Engineer Polyploid Genomes*, n.d.). However, researchers were unable to generate null diploid *Slosd1* mutants. This was hypothesized to result from Arabidopsis and rice having two *OSD1*-like genes, while tomato possesses only a single *OSD1* gene (Underwood & Mercier, 2022), which appears to be essential for mitotic division. To overcome this, *TARDY ASYNCHRONOUS MEIOSIS* (*TAM)* was targeted, another gene which when mutated causes the skipping of meiosis II. This approach was successful, however, the resulting plants produced smaller fruits with fewer seeds than wild-type plants. Moreover, a higher proportion of seeds were underdeveloped 11–42% compared to only 1% in wild-type controls (*Wang et al - 2024 - Harnessing Clonal Gametes in Hybrid Crops to Engineer Polyploid Genomes*, n.d.).

TAMfunctions by regulating THREE DIVISION MUTANT1 (TDM1), which is a component of the anaphase-promoting complex (APC/C) that promotes the termination of meiosis (Cifuentes et al., 2016a). During the first meiotic division, the Cyclin-Dependent Kinase A;1–TAM complex inhibits TDM1 through phosphorylation, preventing premature activation of the APC/C and allowing progression to the second meiotic division. Mutations in the phosphorylation site of *TDM1* in *Arabidopsis* resulted in the skipping of meiosis II, similar to *osd1* or *tam* mutants (Cifuentes et al., 2016b).

## A diagram of a cell cycle AI-generated content may be incorrect.Parthenogenesis

Parthenogenesis, which literally means “from virgin origin”, refers to the development of an embryo from an egg cell without fertilization (see Figure 3). Clonal seed production relies on the absence of a male genetic contribution to the zygote. Several genes, including *BABY BOOM* *(BBM)*, *PARTHENOGENESIS (PAR)*, *WUSCHEL* (*WUS)*, have been identified to induce parthenogenesis when expressed in the egg cell across various species (Conner et al., 2015; Huang et al., 2025; Underwood et al., 2022). The recently discovered *PAR* gene plays an important role in parthenogenesis in naturally apomictic dandelions (Underwood et al., 2022). Deletion of *PAR* leads to loss of parthenogenesis in these apomictic dandelions. Moreover, heterologous expression of the dandelion *PAR* gene in egg cells has successfully induced parthenogenesis in lettuce (Underwood et al., 2022), foxtail millet (Huang et al., 2024), and rice (Dan et al., 2024). Recent studies have shown that combining *MiMe* with *OsBBM1* or *ToPAR* successfully induces synthetic apomixis in rice (Khanday et al., 2019; *Wang et al - 2024 - Harnessing Clonal Gametes in Hybrid Crops to Engineer Polyploid Genomes*, n.d.).

**Figure 3. Sexual reproduction vs Parthenogenic reproduction [3]**. In sexual reproduction (left), the mother cell undergoes meiosis, producing a haploid gamete. This female gamete is fertilized by a male gamete producing a hybrid embryo. In parthenogenic reproduction (right), the mother cell undergoes meiosis, producing a haploid gamete. This female gamete is not fertilized by a male gamete resulting in a haploid embryo.

## A close up of a flower AI-generated content may be incorrect.Approach and rationale

The combination of genetic modifications necessary for inducing apomixis in *Solanum lycopersicum* (Micro-Tom) is complex. In this study, we evaluate genetic modifications designed to skip meiosis II and complete apomeiosis, alongside a modification that is designed to induce parthenogenesis.

Within apomeiosis, this study investigates alternatives to *tam* in the *MiMe* line for Micro-Tom by exploring mutations in *OSD1* and *TDM1*. As previously mentioned, the knockoutof *OSD1* in Micro-Tom results in non-viable plants (*Wang et al - 2024 - Harnessing Clonal Gametes in Hybrid Crops to Engineer Polyploid Genomes*, n.d.). To circumvent this, CRISPR will be used to target the 5' UTR of *OSD1* with the aim of downregulating its expression. Partial downregulation may allow the plant to remain viable while enabling the skipping of meiosis II. An alternative approach involves introducing a mutation into the phosphorylation site of *TDM1.* Two constructs are tested based on this approach: *SlTDM1-P19L* and the in Arabidopsis established *AtTDM1-P17L*(Cifuentes et al., 2016b)*.* These modifications are expected to have a similar effect as a *tam* knock-out mutation, since in both cases TDM1 is not phosphorylated. All three approaches are anticipated to result in the skipping of meiosis II.

**Figure 4. Wild-type Micro-tom.**

The success of these genetic modifications in skipping meiosis II and their impact on fertility are evaluated by assessing pollen size, pollen viability, and seed set. Normal pollen are haploid, whereas pollen that have skipped meiosis II are diploid and therefore larger, making pollen size an indicator of apomeiosis (*Wang et al - 2024 - Harnessing Clonal Gametes in Hybrid Crops to Engineer Polyploid Genomes*, n.d.). The combination of pollen viability and seed set will be used as a measure of fertility.

The dandelion *PAR* gene, driven by an egg-cell-specific promoter is introduced into Micro-Tom to induce parthenogenesis. As previously mentioned, PAR has been shown to be effective at inducing parthenogenesis in lettuce (Underwood et al., 2022), foxtail millet (Huang et al., 2024), and rice (Dan et al., 2024). This study aims to determine its effect in Micro-Tom. The combination of pollen viability and seed set will be used as a measure of fertility.

To further investigate the effects of the PAR gene, an additional genetically modified line expressing red fluorescent protein will be generated to enable the identification of parthenogenetic offspring. This *OLEO-RFP* line will be used as to pollinate potentially parthenogenetic plants. Following fertilization, embryos formed through sexual reproduction will express Red Fluorescent Protein (RFP), whereas embryos formed through parthenogenesis will have lower RFP expression, with fluorescence restricted to the endosperm. Therefore, differences in red fluorescence intensity will enable the discrimination of successful parthenogenetic plants.

## Aims and hypotheses

This study aims to evaluate the effect of two constructs (*SlTDM1*-*P19L and* *AtTDM1*-*P17L* with the potential to skip meiosis II and complete the *MiMe* system. Furthermore, it aims to evaluate the effect of one construct (ToPAR) with the potential to induce parthenogenesis in Micro-Tom. Additionally, we are currently performing transformations for the 5' UTR *osd1* and *OLEO*-*RFP* constructs, which will serve as a resource for future research. We hypothesize that mutations in *SlTDM1*-*P19L* or *AtTDM1*-*P17L* will skip meiosis II, resulting in the production of diploid pollen. Failure to suppress second division would lead to haploid or non-viable pollen. Whereas partial suppression may result in a mixture of diploid and haploid pollen. To test these hypotheses, we assess seed set, pollen size, and pollen viability. We hypothesize that expression of the *PAR* gene in the egg cell will induce embryo formation without fertilization, resulting in haploid embryos. Failure to induce parthenogenesis would lead to continued sexual reproduction and the formation of diploid embryo cells. To test these hypotheses, we assess seed set and pollen viability. Transformants carrying the 5' UTR *osd1* and *OLEO-RFP* constructs will be genotyped by PCR to confirm successful integration. Establishing apomeiosis and parthenogenesis in tomato would represent an important advance, with potential applications in clonal seed production and hybrid breeding.

# Methods

## Genetic material

The genes and mutations referred to in this study are *spo11-1*, *rec8*, *tam (MiMe),* CRISPR 5’UTR *OSD1, pOLEOSIN::RFP, SlTDM1–P19L, AtTDM1–P17L, pEC1.1::ToPAR.*

## Plant material

All experiments were performed using the tomato cultivar Micro-Tom (*Solanum lycopersicum)*. Micro-Tom is well suited for plant transformations due to its compact size, rapid growth cycle, and susceptibility to genetic manipulation.

## Tomato transformation

**Constructs**Two independent Agrobacterium-mediated transformations were performed on Micro-Tom. First, a CRISPR/Cas12a targeting the 5' untranslated region (5' UTR) of *SlOSD1*, along with the *35S::GFP* (green fluorescent protein) and *pNOS::NPTII* (kanamycin resistance)selection markers, were introduced. Second, a *pOLEO::OLEO-RFP* construct, containing the same markers was introduced.

A diagram of a plant life cycle

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**Figure 5. Overview of tomato transformation.** Wild-type Micro-Tom seeds are first sterilized, after which they are sowed in GEM. Simultaneously Agrobacterium culture is continuously transferred and incubated. 7-8 days after sowing the cotyledons are cut and transformed by the agrobacterium. After transformation the explants are transferred to COM plates. After two days the explants are transferred to POM plates. After three days the explants are transferred to SIM plates. After two weeks the explants are transferred to SIM+ plates, which are refreshed every two weeks. Once shoots develop, the explant is cut and transferred to RIM boxes. After root formation, the plant can be transferred to soil and genotyped.

**Seed sterilization and germination**

Seeds were surface sterilized in a flow cabinet using 1% (v/v) chlorine solution (prepared by diluting commercial bleach 1:4) for 20 minutes on a rolling tray. Tweezers were sterilized with 70% ethanol and flamed prior to use. Seeds were rinsed three times in sterile Mili-Q. Sterilised seeds were sown on germination media consisting of Murashige and Skoog (MS) salts with vitamins (2.2 g/L), Sucrose (10 g/L), Daishin agar (8 g/L), adjusted to pH 5.8. Seeds were gently pressed into the surface, allowing contact with air. Germination boxes were incubated in a climate chamber (24°C, 16 hrs light/8hrs dark).

**Agrobacterium culture**

Agrobacterium tumefaciens strains containing the respective constructs were grown from fresh plates in 5mL LB medium supplemented with Rifampicin (20 mg/L), Kanamycin (100 mg/L), and Gentamicin (10 mg/L) at 28 °C overnight with shaking. The next day, 0.1 mL of culture was inoculated into 10 mL fresh LB medium with Rifampicin (20 mg/L) and Kanamycin (100 mg/L) and incubated overnight under the same conditions.

**Dissection of cotyledons**

Cotyledons were dissected from 7-8 days old seedlings under sterile conditions (downflow cabinet). Hypocotyls were cut using sterile scissors to isolate rectangular cotyledon explants. Explants were placed adaxial side up on sterile filter paper soaked in liquid inoculation medium (LIM) consisting of Murashige and Skoog (MS) salts without vitamins (4.4 g/L), Vitamins Gamborg B5 (112 mg/L), Sucrose (30 g/L), MES buffer (0.5 g/L), adjusted to pH 5.8. Next, explants were transferred to co-cultivation medium (COM) plates consisting of Murashige and Skoog (MS) salts without vitamins (4.4 g/L), Vitamins Gamborg B5 (112 mg/L), Sucrose (30 g/L), MES buffer (0.5 g/L), Daishin agar (8 g/L), adjusted to pH5.8. Following autoclaving, the medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.05 mg/L), zeatin (2.0 mg/L), and acetosyringone (200 μM). The plates were incubated overnight in the climate chamber (24 °C, 16 hrs light/8 hrs dark), covered with filter paper to decrease light intensity.

**Transformation and co-cultivation**

Agrobacterium (1mL) was inoculated in 25mL LB medium with antibiotics (Kanamycin and vancomycin). Next, the Agrobacterium was incubated at 28°C with shaking until an OD600 = 0.2-0.4 was reached. Cells were pelleted at 3000 rpm, resuspended in 20mL LIM containing Acetosyringone (200μM) and incubated at room temperature for 1 hour. Explants were collected from COM plates and washed in LIM, then incubated with the Agrobacterium suspension for 20 minutes on a rotary shaker. Following infection, explants were washed once with fresh LIM and transferred adaxial side up on COM. COM plates were incubated for two days in the climate chamber covered with a storage container to block light (24 °C, 24 hrs dark).

**Transfer to post co-culture medium (POM)**

Explants were transferred to post co-culture medium (POM) consisting of Murashige and Skoog (MS) salts without vitamins (4.4 g/L), Vitamins Gamborg B5 (112 mg/L), Sucrose (30 g/L), ethane sulfonic acid buffer (0.5 g/L), Daishin agar (10 g/L), adjusted to pH 5.8. Following autoclaving, the medium was supplemented with zeatin (2.0 mg/L), Cefotaxime (200 mg/L), Vancomycin (50 mg/L). Plates were incubated in the climate chamber for three days with filter paper on top to reduce light intensity (24 °C, 16 hrs light/8 hrs dark). Plates were sealed with Leukopor.

**Transfer to Shoot Inducing Medium (SIM)**

Five days after infection explants were transferred to shoot inducing medium (SIM) consisting of Murashige and Skoog (MS) salts without vitamins (4.4 g/L), Vitamins Gamborg B5 (112 mg/L), Glucose (10 g/L), ethane sulfonic acid buffer (0.5 g/L), Daishin agar (8 g/L), adjusted to pH5.8. Following autoclaving, the medium was supplemented with zeatin (2.0 mg/L), Carbenicillin (500 mg/L), Kanamycin (100 mg/L). Leaves were gently pressed into medium, adaxial side of leave up. Plates were sealed with double layer of Leukopor and in incubated in culture room for two weeks (24 °C, 16 hrs light/8 hrs dark).

**Transfer to SIM+**

Explants were transferred to fresh SIM+ consisting of SIM supplemented with IAA (0.1 mg/L), adaxial side of leave up. Necrotic tissue (brown) was removed. Calluses with emerging shoots were transferred to root inducing medium (RIM). Media were refreshed and calluses cleaned every two weeks.

**Transfer to Root Inducing Medium (RIM)**

Shoots were cut and inserted 1-2 mm into root inducing medium (RIM) consisting of Murashige and Skoog (MS) salts with vitamins (4.4 g/L), Sucrose (15 g/L), Daishin agar (6 g/L), adjusted to pH 6.0. Following autoclaving, the medium was supplemented with Indole-3-Butyric acid (IBA) (0.25 mg/L), Cefotaxime (200 mg/L), Vancomycin (100 mg/L). Cultures were incubated in the climate chamber (24 °C, 16 hrs light/8 hrs dark) for 2-4 weeks until roots developed.

**Genotyping (only SlOSD1 5UTR)**

Fresh young leaves (at least 2 mm in diameter) were obtained from the Micro-Tom plants and transferred into 20 μL dilution buffer. Leaves were macerated with a 100 μL pipette tip until the solution turned green. The homogenate was centrifuged briefly to pellet debris, and 0.5 μL of the supernatant was used as the template for the PCR.

PCR reactions were performed in a total volume of 10 μL containing: 5 μL of 2× reaction buffer (containing polymerase, dNTPs, and MgCl₂), 0.5 μL each of forward and reverse primers (10 μM), 3.5 μL of Mili-Q, and 0.5 μL of template. Reactions were prepared on ice.

SlOSD1 5UTR Forward primer: 5’GTCACGTGCTTCACCATTTTCA-3’

SlOSD1 5UTR Reverse primer: 5’GTCTCTTCCTTCGGCCATTTTG-3’

Thermal cycling was performed under the following conditions: Initial denaturation at 98 °C for 5 min; followed by 40 cycles of denaturation at 98 °C for 5 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 30 sec; with a final extension at 72 °C for 1 min. Reactions were held at 4 °C until analysis. Analysis was performed in a 1% agarose gel.

## Flow cytometry

Flow cytometry was used to analyse the pollen viability from the SlTDM1-P19L and AtTDM1-P17L lines. Flowers were collected at the pre-anthesis stage, petals were still closed or just beginning to open. Anther segments were isolated and divided into four parts. These parts were vortexed, after which AmphaFluid was added. This mixture was vortexed again, followed by filtration through a (50 µm) mesh prior to analysis using the Amphasys flow cytometer. A gating was created within the 18 MHz plot on a wildtype measurement. This gating was used to select all viable pollen and was consistently applied across all samples.

**Negative control.** Wildtype Micro-Tom pollen.

**Positive control.** *MiMe* Micro-Tom pollen (*spo11-1, rec8, tam*).

## Seed set

Seed sets were analysed in Micro-Tom lines expressing SlTDM1-P19L, AtTDM1-P17L and ToPAR, alongside wildtype as a negative control and *MiMe* (*spo11-1, rec8, tam*) as a positive control.

For each genotype, seeds were counted from 10 fruits per plant. The total number of seeds per fruit was recorded to assess fertility.

## Pollen staining

Pollen size was assessed in Micro-Tom lines transformed with the *SlTDM1-P19L* and *AtTDM1-P17L* constructs. Wildtype Micro-Tom was used as a negative control, and *MiMe* Micro-Tom (*spo11-1, rec8, tam*), was used as a positive control.

Floral buds were collected at early developmental stages, when petals were still closed or just beginning to open and immediately fixed in Carnoy’s fixative (ethanol:chloroform:glacial acetic acid, 6:3:1, v/v/v). Anthers were dissected, cut transversely and a thin slice was removed. Pollen were stained with alexander staining consisting of 96% ethanol (10% v/v), malachite green (0.01% w/v in ethanol), distilled water (54.5% v/v), glycerol (25% v/v), acid fuchsin (0.05% w/v in water), Orange G (0.005% w/v in water), and glacial acetic acid (4% v/v). The solution was stored in the dark to preserve dye stability. An 8 μL droplet of Alexander staining was placed on a microscope slide. Next the slice of anther was immersed in the Alexander staining. Pollen grains were released by gentle tapping with tweezers. A coverslip was applied, after which samples were immediately imaged using bright field microscopy. Pollen diameter was measured using ImageJ.

**Cell pose and ImageJ analysis**

Pollen diameter was analysed with a combination of cell pose and ImageJ. A custom plugin developed at Radboud University enabled integration of Cell pose outputs into ImageJ for automated measurement of the pollen number, diameter, and staining intensity. Manual measurements were made to confirm the automated measurements (see Figure 16 in Appendix), there was a shift downward in pollen size when compared to automated.

# Statistics

Pollen diameter, pollen viability and seed set measurements were tested for normality using the Shapiro-Wilk test and all datasets showed a non-normal distribution (for results see <https://github.com/MaartenWessel/Apomixis>). Since the datasets were not normally distributed, Mann-Whitney U tests were used for statistical comparisons.

# Results

This study investigated the effects of two constructs designed to introduce the skipping of meiosis II (*SlTDM1-P19L* and *AtTDM1-P19L*), and one construct designed to induce parthenogenesis (*ToPAR*). Lastly, two constructs, CRISPR targeting of 5' UTR *OSD1* and *pOLEO::RFP,* have been transformed into Micro-Tom.

## Skipping meiosis II (*SlTDM1-P19L*)

To assess the impact of the *SlTDM1-P19L* mutation on the skipping of meiosis II, pollen diameter was analysed (see Figure 6), as pollen diameter corelates with ploidy (Wang et al., 2024). In total, one flower bud was analysed for each of the 71 transgenic plants which were measured. The pollen were stained and imaged, after which automatic detection and size measurement were performed on these images. None were significantly the same as the wild-type. On average, 16,684 pollen grains were analysed per plant. The wild-type had a mean pollen diameter of 22.14µm, while the *MiMe* had a mean pollen diameter of 29.30 µm. Across all samples, pollen diameters ranged from 1.37 µm to 209.03 µm. Only two lines were significantly the same as the *MiMe* control (C7P1 and C4P1), suggesting that these skipped meiosis II. Additionally, four lines (C4P4, C18P1, C24P1, C31P1) showed two distinct pollen diameter groups (see figure 7), which is an indication that there are haploid and diploid pollen present. This pattern suggests that these partially skipped meiosis II.

To assess the impact of the *SlTDM1-P19L* mutation on the fertility, pollen viability was analysed using the Amphasys flow cytometer (see Figure 8). Pollen viability was measured using 3–5 biological replicates per plant, with an average of 8994 pollen grains analysed per replicate. In total, 38 of the 71 transgenic plants were analysed. Among these, 26 plants displayed significantly reduced pollen viability compared to wild-type. Notably, 10 lines had a pollen viability below 40%, substantially lower than the wild-type (mean viability 89.61%, n = 6).

To further assess the impact of the *SlTDM1-P19L* mutation on fertility, seed set was analysed (see Figure 9) Seed set was measured in 10 fruits per plant. In total, 20 transgenic plants were analysed. Among these, 14 displayed a significantly reduced seed set compared to wild-type. The wild-type had a mean seed set of 46.8 seeds per fruit (standard deviation 24.97; range 12–96).

A graph with lines and dots

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**Figure 6. SlTDM1-P19L pollen diameter.** Pollen diameter samples of 71 SlTDM1-P19L plants, presented in violin plots. Samples were ordered by average pollen diameter from long to short. Wild-type (red) serves as the negative control. MiMe (green) serves as the positive control. Each bar was labelled with the callus number “C” and plant number “P”, second measurements are indicated with “-2”. Significant differences were determined by the Mann-Whitney U test. Samples not significantly different compared to wild-type (P ≥ 0.05), were labelled with a red "A". Samples not significantly different compared to MiMe (P ≥ 0.05), were labelled with a green "B". Samples that differed significantly compared to both wild-type and MiMe (P < 0.05), were labelled with a blue “C”.

A graph of different colored lines

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**Figure 7. SlTDM1-P19L pollen diameter.** This figure shows only the six most interesting samples present in Figure 6, based on the appearance of two separated pollen diameter groups. Labels are the same as in Figure 6. Significant differences were determined by the Mann-Whitney U test. Samples not significantly different compared to wild-type (P ≥ 0.05), were labelled with a red "A". Samples not significantly different compared to MiMe (P ≥ 0.05), were labelled with a green "B". Samples that differed significantly compared to both wild-type and MiMe (P < 0.05), were labelled with a blue “C”.

A graph with blue and black lines

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**Figure 8. SlTDM1-P19L pollen viability.** Pollen viability percentage of 38 SlTDM1-P19L plants. Samples were ordered by average pollen viability percentage from highest to lowest. Wild-type (red) serves as negative control Each bar was labelled with the callus number “C” and plant number “P”. Sample sizes range from 3-5 per plant. Boxplots display the median (centre line), interquartile range (box), and full data range (whiskers). Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by a star (P < 0.05).

A graph of seed set

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**Figure 9. SlTDM1-P19L seed set.** Average number of seeds per fruit of 21 SlTDM1-P19L plants. Samples were ordered by average number of seeds per fruit from highest to lowest. Wild-type (red) serves as the negative control. Each bar was labelled with the callus number “C” and plant number “P”. Seed sets were measured in 10 fruits per plant. Values are displayed as mean ± s.d. in the bar plot. Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by one star (P < 0.05) or three stars (P. < 0.001).

## Skipping meiosis II (*AtTDM1-P17L*)

To assess the impact of the *AtTDM1-P17L* mutation on the skipping of the meiosis II in these tomato lines, pollen diameter was analysed using the same approach as for the *SlTDM1-P19L* lines (see Figure 10). In total, 9 transgenic plants were analysed. All displayed a significant difference in diameter compared to wild-type. On average 26,655 pollen grains were analysed per plant. The wild-type had a mean pollen diameter of 22.14 µm, while the *MiMe* had a mean pollen diameter of 29.30 µm. Across all samples, pollen diameters ranged from 1.37 µm to 156.36 µm. None of the lines showed two distinct pollen diameter groups.

To assess the impact of the *AtTDM1-P17L* mutation on the fertility, pollen viability was analysed (see Figure 11). Pollen viability was measured using 3–5 biological replicates per plant, with an average of 9587 pollen grains analysed per replicate. In total six transgenic plants were analysed. Among these, three displayed a significantly reduced pollen viability compared to wild-type. Wild-type had a mean pollen viability of 89.61% (n = 6).

To further assess the impact of the *AtTDM1-P17L* mutation on fertility, seed set was analysed (see Figure 12). Seed set was measured in ten fruits per plant. In total five transgenic plants were analysed. Among these, three displayed a significantly reduced seed set compared to wild-type. The wild-type had a mean seed set of 46.8 seeds per fruit (standard deviation 24.97; range 12–96).

A diagram of pollen diameter

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**Figure 10. AtTDM1-P17L pollen diameter.** Pollen diameter samples of four AtTDM1-P17L plants, presented in violin plots. Samples were ordered by average pollen diameter from long to short. Wild-type (red) serves as the negative control. MiMe (green) serves as positive control. Each bar was labelled with the callus number “C” and plant number “P”. Significant differences were determined by the Mann-Whitney U test. Samples not significantly different compared to wild-type (P ≥ 0.05), were labelled with a red "A". Samples not significantly different compared to MiMe (P ≥ 0.05), were labelled with a green "B". Samples that differed significantly compared to both wild-type and MiMe (P < 0.05), were labelled with a blue “C”.

A graph of a number of cells

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**Figure 11. AtTDM1-P17L pollen viability.** Pollen viability percentage of 6 AtTDM1-P17L plants, samples were ordered by average pollen viability percentage from highest to lowest. Wild-type (red) serves as negative control. Each bar was labelled with the callus number “C” and plant number “P”. Sample sizes range from 3-5 per plant. Boxplots display the median (centre line), interquartile range (box), and full data range (whiskers). Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by a star (P < 0.05).

A graph of seed set

AI-generated content may be incorrect.

**Figure 12. AtTDM1-P17L seed set.** Average number of seeds per fruit of four AtTDM1-P17L plants, samples were ordered by average number of seeds per fruit from highest to lowest. Wild-type (red) serves as the negative control. Each bar was labelled with the callus number “C” and plant number “P”. Seed sets were measured in 10 fruits per plant. Values are displayed as mean ± s.d. in the bar plot. Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by one star (P < 0.05) or three stars (P. < 0.001).

## Inducing parthenogenesis (*ToPAR*)

To assess the impact of the *ToPAR* induction on the fertility, pollen viability was evaluated analysed (see Figure 13). Pollen viability was measured using 3–5 biological replicates per plant, with an average of 10,061 pollen grains analysed per replicate. In total, 95 transgenic plants were analysed. Among these, 47 displayed a significantly reduced pollen viability and 3 displayed a significantly increased pollen viability compared to wild-type. Notably, 5 lines had a pollen viability below 40%. Wild-type had a mean viability of 89.61% (n=6).

To further assess the impact of the *ToPAR* induction on fertility, seed set was analysed (see Figure 14). Seed set was measured in ten fruits per plant. In total seven transgenic plants were analysed. Among these, 5 displayed a significantly reduced seed set compared to wild-type. The wild-type had a mean seed set of 46.8 seeds per fruit (standard deviation 24.97; range 12–96).

A graph of a graph

AI-generated content may be incorrect.

**Figure 13. ToPAR pollen viability.** Pollen viability percentage of 95 ToPAR plants. Samples were ordered by average pollen viability percentage from highest to lowest. Wild-type (red) serves as negative control. Each bar was labelled with the callus number “C” and plant number “P”. Sample sizes range from 3 to 5 per plant. Boxplots display the median (centre line), interquartile range (box), and full data range (whiskers). Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by a star (P < 0.05).

A graph showing different types of seeding

AI-generated content may be incorrect.

**Figure 14. ToPAR seed set.** Average number of seeds per fruit of 7 ToPAR plants. Samples were ordered by average number of seeds per fruit from highest to lowest. Wild-type (red) serves as the negative control, with an average seed set of 46.8 seeds per fruit (s.d. 24.97, n = 10). Each bar was labelled with the callus number “C” and plant number “P”. Seed sets were measured in 10 fruits per plant. Values are displayed as mean ± s.d. in the bar plot. Significant differences compared to wild-type were determined by Mann-Whitney U test and are indicated by one star (P < 0.05) or three stars (P. < 0.001).

## Transformations

A collage of photos of plants

AI-generated content may be incorrect.To explore alternatives to skipping meiosis II and identifying parthenogenesis, two constructs were transformed into tomato. Most of the *OSD1* transformed plants did not show GFP in the roots, which indicates that these transformation were unsuccessful or that there is a silencing of GFP. However, multiple plants did show clear GFP in the roots, indicating successful transformation events. Due to time restraints these plants have not been genotyped yet. The *OLEO-RFP* transformed plants were at the transferring to RIM stage. No roots had formed yet. Plants have not been genotyped yet.

**Figure 15. Transformation of CRISPR 5’ UTR osd1.** A. shows the germination of the seeds. B. shows the explants on co-culture medium. C. shows the explants on SIM medium, shoots have developed. D. shows the extracted shoots in RIM medium. E. shows green fluorescence coming from the roots. F. shows the first plants transferred to pots. G. shows the plants after two weeks.

# Discussion

## Skipping meiosis II

To asses the effect of the *SlTDM1-P19L* and the *AtTDM1-P17L* mutations on skipping meiosis II pollen diameter was analysed. Furthermore, to assess the fertility the pollen viability and seed set were analysed. Pollen diameter measurements in the *SlTDM1- P19L* lines suggest that six plants have formed diploid pollen, indicating that meiosis II was partially skipped. Pollen diameter measurements in the *AtTDM1-P17L* lines suggest that no plants formed diploid pollen, indicating that meiosis II was not skipped. Fertility was lowered in both *SlTDM1-P19L* and *AtTDM1-P17L*.

*SlTMD1* appears to induce diploid pollen formation in plants C7P1 and C4P1. Both of these plants have a pollen diameter statistically similar to the *MiMe* positive control. Furthermore, the larger pollen population has a diameter of ~32 µm, which is in line with earlier research (Wang et al., 2024). Second measurements of C7P1 and C4P1 (C7P1-2, C4P1-2) differ notably from the first measurements and the MiMe positive control. These first measurements were not exposed to Carnoy’s fixative whereas the second once were. We hypothesize that the ethanol present within Carnoy’s fixative, has caused slight dehydration (Ermolaev et al., 2024), resulting in reduced pollen size. An important sidenote is that the Wildtype control, MiMe control and measurements: “ C3P1, C4P1, C6P1, C7P1” have not been exposed to Carnoy’s fixative.

Notably, the plants C4P4, C18P1, C24P1 and C31P1 show two distinct pollen diameter populations, which suggests presence of diploid pollen. However, the larger pollen population does not reach the size of the larger pollen in the *MiMe* control. According to Yazong (Wang et al., 2024), haploid pollen range from 20.03–28.15 µm, while diploid pollen range from 28.15–40.23 µm. The larger pollen from C4P4 and C18P1 fall between 25-30 µm, and therefore partially qualify based on Yazong’s standard. The larger pollen population in C24P1 and C31P1 fall between 20-25 µm and therefore cannot be classified as diploid pollen based on Yazong’s standard. However, the presence of two distinct pollen size populations is unlikely to stem from only haploid pollen. We hypothesize that overall pollen size is reduced as a result from dehydration during storage of the flower buds in Carnoy’s fixative (Ermolaev et al., 2024). This would also explain why the smaller populations present in these measurements are smaller than the wild-type (haploid) pollen.

An alternative explanation for these smaller pollen populations would be the formation of aneuploid cells. Aneuploid cells can form due to an error during chromosome segregation in meiosis I (Compton, 2011). This results in cells which have incomplete chromosome sets and are generally smaller and less viable (Henry et al., 2010).

A close-up of a microscope

AI-generated content may be incorrect. A limitation that must be addressed is that the automatic pollen analysis includes measurements of dying or dead pollen which have not fully collapsed. These appear visually smaller (see Figure 16). Unfortunately, this could bias the average pollen diameter, especially in plants which have a lower pollen viability. To mitigate this, diameter measurements should be filtered to only include viable pollen. This is possible using the images already collected, as the Alexander staining used in this study stains viable pollen uniformly purple-red and non-viable pollen blue-green. Pollen grains which are starting to degrade often still have a purple nucleus.

**Figure 16. Pollen diameter image (SlTDM1-P19L C12P7).** Viable pollen are uniformly dark purple, non-viable pollen are blue-green, pollen which start degradation still show a purple nucleus. Degrading pollen are visibly smaller.

A manual validation of the automatically collected pollen diameters was performed to check for accuracy (see Appendix Figure 17). Manual data showed a shift downwards compared to automatic data. The overall shape of the violin plots is visually similar. While this raises uncertainty about the reliability of either method, the manual validation was included for transparency. Due to limited experience with manual and automatic pollen diameter measurements, it is unclear whether the differences stem from a systematic error in the automatic pipeline or an inconsistency in the manual approach.

Fertility is lowered in ~70% of *SlTDM1* plants (pollen viability 68% and seed set 70%) and 50-60% of *AtTDM1* plants (pollen viability 50% and seed set 60%). Likely this is partially a result from the tissue culture and transformation side effects (Ellul et al., 2003) or formation of aneuploid cells (Henry et al., 2010). When pollen viability and seed set measurements are compared no clear pattern emerges. When only the seed set of the plants which are likely to have diploid pollen (C18P1 and C31P1) are analysed, the average seed set is 7.7 seeds per tomato, which is 16.45% of the wild-type seed set.

Pollen viability measurements can be improved by including a new wild-type sample in each measurement session. Pollen viability samples were collected and measured over five weeks, at different times and temperatures. This relatively long measurement period was due to asynchronous flowering and shared access to the impedance flow cytometer. Fluctuations in temperature are known to influence pollen viability (Iovane & Aronne, 2022; Pressman, 2002). Taking new wild-type samples for every measurement would allow the normalization of the measurements.

Since only 8 plants of the *AtTDM1-P17L* line have been (partially) analysed, it cannot be ruled out that the insertion of the mutated *AtTDM1-P17L* may result in the skipping of meiosis II. To confirm or disprove this, additional data, specifically pollen diameter measurements, are required.

## Parthenogenesis

To assess the effect of the *ToPAR* induction on the fertility, pollen viability was evaluated analysed. Overall fertility was lowered. When the pollen viability and seed set measurements of *ToPAR* plants are compared, a pattern emerges. Plants with normal pollen viability (e.g. C32P1) also have a normal seed set. Plants with a reduced pollen viability also have a reduced seed set (e.g. C27P1, C19P1). Plants with strongly reduced pollen viability also have a strongly reduced seed set (e.g. C1P5, C20P1, C21P1). The *ToPAR* construct is driven by an egg cell-specific promoter, making it unlikely that the reduced fertility is caused by *ToPAR* expression, as the gene would not be expressed in the pollen. It is more likely that the reduced fertility is a side effect of the tissue culture, as these plants are in the first generation after transformation. Transformations in tomato can lead to an increase in ploidy (Ellul et al., 2003). Tetraploidy has been reported to decrease fertility (Nilsson, 2009, 2010).

## Transformations

To explore alternatives to skipping meiosis II and identifying parthenogenesis, two constructs were transformed into tomato. Both transformations were not completed. However, multiple *osd1* transgenic plants have roots which show GFP.

Unfortunately, most of the transformed plants did not show GFP expression in the roots. This was unexpected given that the construct included the selective marker *pNOS::NPTII,* providing kanamycin resistance, and kanamycin was present in the SIM. One possible explanation is that transformation occurred in cells that came in direct contact with the media, while shoots developed from untransformed cells which were not in contact with the media. As a result, untransformed cells evaded selection, leading to the development of shoots lacking the construct.

Tissue culture and transformation seems to have affected the fertility of the plants. To get a better understanding of the real fertility, second generation plants should be used. This is often a problem due to plants growing slowly and a second generation will take time. One thing which can be done to improve the situation is to screen for tetraploid plants. This can be done either through fluorescent DAPI staining in combination with flow cytometry (Smulders et al., 1994), or by determining the starch concentration which is likely higher in tetraploid cells (Miller et al., 2012).

## Future research

In future transformation ploidy analysis through flow cytometry or through starch concentration analysis will be performed after transformation to determine ploidy. This way potential tetraploid plants can be excluded for fertility analysis. Already collected seeds will be cleaned, after which seed size will be analysed, which can also give an indication of ploidy. Additionally, germination assays can be performed to further assess fertility.

Mutations induced by the CRISPR construct targeting the 5’UTR *OSD1* and the transformation success of the *OLEOSIN-RFP* lines will be confirmed through PCR analysis. Similarly to what was done in this research, the effect of the CRISPR 5’UTR *OSD1* mutation on skipping meiosis II will be determined through pollen diameter analysis, and fertility will be analysed through pollen viability and seed set. The *OLEOSIN-RFP* will be used to screen *ToPAR* plants for parthenogenesis. *ToPAR* plants will be pollinated with the *OLEOSIN-RFP* pollen, the strength of the RFP signal in the seeds will be used to determine whether the seeds are a result of sexual fertilization or parthenogenesis.

When apomeiosis and parthenogenesis have successfully been established in tomatoes, the logical next step is to engineer autonomous endosperm. Until now all synthetic apomictic plants form sexual endosperms. The major downside of this is that the induction of parthenogenesis and the fertilization of the central cell has to occur simultaneously. *FERTILIZATION-INDEPENDENT SEED (FIS)* mutants have been used to create autonomous endosperm in *Arabidopsis*, but embryo development was not successful (Chaudhury et al., 1997; Kiyosue et al., 1999; Ohad et al., 1996).

# Conclusion

*SlTDM1* expression in Micro-Tom induces partial skipping of meiosis II, leading to larger, likely diploid pollen in several lines, fertility is reduced. *AtTDM1* does not appear to induce diploid pollen formation and also shows reduced fertility. *ToPAR* lines show a lowered fertility, which is likely more due to the tissue culture than the expression of the *ToPAR* gene. Further analysis in second-generation plants and ploidy assessments will further elucidate the effect of these genetic modifications.

# References

Bai, Y., & Lindhout, P. (2007). Domestication and Breeding of Tomatoes: What have We Gained and What Can We Gain in the Future? *Annals of Botany*, *100*(5), 1085–1094. https://doi.org/10.1093/aob/mcm150

Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S., & Peacock, W. J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *94*(8), 4223–4228. https://doi.org/10.1073/pnas.94.8.4223

Cifuentes, M., Jolivet, S., Cromer, L., Harashima, H., Bulankova, P., Renne, C., Crismani, W., Nomura, Y., Nakagami, H., Sugimoto, K., Schnittger, A., Riha, K., & Mercier, R. (2016a). TDM1 Regulation Determines the Number of Meiotic Divisions. *PLOS Genetics*, *12*(2), e1005856. https://doi.org/10.1371/journal.pgen.1005856

Cifuentes, M., Jolivet, S., Cromer, L., Harashima, H., Bulankova, P., Renne, C., Crismani, W., Nomura, Y., Nakagami, H., Sugimoto, K., Schnittger, A., Riha, K., & Mercier, R. (2016b). TDM1 Regulation Determines the Number of Meiotic Divisions. *PLOS Genetics*, *12*(2), e1005856. https://doi.org/10.1371/journal.pgen.1005856

Compton, D. A. (2011). Mechanisms of aneuploidy. *Current Opinion in Cell Biology*, *23*(1), 109–113. https://doi.org/10.1016/j.ceb.2010.08.007

Conner, J. A., Mookkan, M., Huo, H., Chae, K., & Ozias-Akins, P. (2015). A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. *Proceedings of the National Academy of Sciences*, *112*(36), 11205–11210. https://doi.org/10.1073/pnas.1505856112

d’Erfurth, I., Jolivet, S., Froger, N., Catrice, O., Novatchkova, M., & Mercier, R. (2009). Turning Meiosis into Mitosis. *PLoS Biology*, *7*(6), e1000124. https://doi.org/10.1371/journal.pbio.1000124

Dan, J., Xia, Y., Wang, Y., Zhan, Y., Tian, J., Tang, N., Deng, H., & Cao, M. (2024). One-line hybrid rice with high-efficiency synthetic apomixis and near-normal fertility. *Plant Cell Reports*, *43*(3), 79. https://doi.org/10.1007/s00299-024-03154-6

Ellul, P., Garcia-Sogo, B., Pineda, B., Ríos, G., Roig, L., & Moreno, V. (2003). The ploidy level of transgenic plants in Agrobacterium-mediated transformation of tomato cotyledons (Lycopersicon esculentum L.Mill.) is genotype and procedure dependent. *Theoretical and Applied Genetics*, *106*(2), 231–238. https://doi.org/10.1007/s00122-002-0928-y

Ermolaev, A., Mardini, M., Buravkov, S., Kudryavtseva, N., & Khrustaleva, L. (2024). A Simple and User-Friendly Method for High-Quality Preparation of Pollen Grains for Scanning Electron Microscopy (SEM). *Plants*, *13*(15), 2140. https://doi.org/10.3390/plants13152140

Henry, I. M., Dilkes, B. P., Miller, E. S., Burkart-Waco, D., & Comai, L. (2010). Phenotypic Consequences of Aneuploidy in*Arabidopsis thaliana*. *Genetics*, *186*(4), 1231–1245. https://doi.org/10.1534/genetics.110.121079

Hojsgaard, D., Klatt, S., Baier, R., Carman, J. G., & Hörandl, E. (2014). Taxonomy and Biogeography of Apomixis in Angiosperms and Associated Biodiversity Characteristics. *Critical Reviews in Plant Sciences*, *33*(5), 414–427. https://doi.org/10.1080/07352689.2014.898488

Huang, Y., Liang, Y., Xie, Y., Rao, Y., Xiong, J., Liu, C., Wang, C., Wang, X., Qian, Q., & Wang, K. (2024). Efficient haploid induction via egg cell expression of dandelion *PARTHENOGENESIS* in foxtail millet ( *Setaria italica* ). *Plant Biotechnology Journal*, *22*(7), 1797–1799. https://doi.org/10.1111/pbi.14302

Huang, Y., Meng, X., Rao, Y., Xie, Y., Sun, T., Chen, W., Wei, X., Xiong, J., Yu, H., Li, J., & Wang, K. (2025). OsWUS-driven synthetic apomixis in hybrid rice. *Plant Communications*, *6*(1), 101136. https://doi.org/10.1016/j.xplc.2024.101136

Iovane, M., & Aronne, G. (2022). High temperatures during microsporogenesis fatally shorten pollen lifespan. *Plant Reproduction*, *35*(1), 9–17. https://doi.org/10.1007/s00497-021-00425-0

Khanday, I., Skinner, D., Yang, B., Mercier, R., & Sundaresan, V. (2019). A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. *Nature*, *565*(7737), 91–95. https://doi.org/10.1038/s41586-018-0785-8

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J. J., Goldberg, R. B., & Fischer, R. L. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *96*(7), 4186–4191. https://doi.org/10.1073/pnas.96.7.4186

Labroo, M. R., Studer, A. J., & Rutkoski, J. E. (2021). Heterosis and Hybrid Crop Breeding: A Multidisciplinary Review. *Frontiers in Genetics*, *12*, 643761. https://doi.org/10.3389/fgene.2021.643761

Miller, M., Zhang, C., & Chen, Z. J. (2012). Ploidy and Hybridity Effects on Growth Vigor and Gene Expression in *Arabidopsis thaliana* Hybrids and Their Parents. *G3 Genes|Genomes|Genetics*, *2*(4), 505–513. https://doi.org/10.1534/g3.112.002162

Nilsson, E. (2009). THE POTENTIAL FRUIT SIZE OF TETRAPLOID TOMATO. *Hereditas*, *49*(1–2), 237–240. https://doi.org/10.1111/j.1601-5223.1963.tb01877.x

Nilsson, E. (2010). SOME EXPERIMENTS WITH TETRAPLOID TOMATOES. *Hereditas*, *36*(2), 181–204. https://doi.org/10.1111/j.1601-5223.1950.tb03371.x

Ohad, N., Margossian, L., Hsu, Y. C., Williams, C., Repetti, P., & Fischer, R. L. (1996). A mutation that allows endosperm development without fertilization. *Proceedings of the National Academy of Sciences*, *93*(11), 5319–5324. https://doi.org/10.1073/pnas.93.11.5319

Pressman, E. (2002). The Effect of Heat Stress on Tomato Pollen Characteristics is Associated with Changes in Carbohydrate Concentration in the Developing Anthers. *Annals of Botany*, *90*(5), 631–636. https://doi.org/10.1093/aob/mcf240

Smulders, M. J. M., Rus-Kortekaas, W., & Gilissen, L. J. W. (1994). Development of polysomaty during differentiation in diploid and tetraploid tomato (Lycopersicon esculentum) plants. *Plant Science*, *97*(1), 53–60. https://doi.org/10.1016/0168-9452(94)90107-4

Song, M., Wang, W., Ji, C., Li, S., Liu, W., Hu, X., Feng, A., Ruan, S., Du, S., Wang, H., Dai, K., Guo, L., Qian, Q., Si, H., & Hu, X. (2024). Simultaneous production of high-frequency synthetic apomixis with high fertility and improved agronomic traits in hybrid rice. *Molecular Plant*, *17*(1), 4–7. https://doi.org/10.1016/j.molp.2023.11.007

Underwood, C. J., & Mercier, R. (2022). Engineering Apomixis: Clonal Seeds Approaching the Fields. *Annual Review of Plant Biology*, *73*(1), 201–225. https://doi.org/10.1146/annurev-arplant-102720-013958

Underwood, C. J., Vijverberg, K., Rigola, D., Okamoto, S., Oplaat, C., Camp, R. H. M. O. D., Radoeva, T., Schauer, S. E., Fierens, J., Jansen, K., Mansveld, S., Busscher, M., Xiong, W., Datema, E., Nijbroek, K., Blom, E.-J., Bicknell, R., Catanach, A., Erasmuson, S., … Van Dijk, P. J. (2022). A PARTHENOGENESIS allele from apomictic dandelion can induce egg cell division without fertilization in lettuce. *Nature Genetics*, *54*(1), 84–93. https://doi.org/10.1038/s41588-021-00984-y

Vernet, A., Meynard, D., Lian, Q., Mieulet, D., Gibert, O., Bissah, M., Rivallan, R., Autran, D., Leblanc, O., Meunier, A. C., Frouin, J., Taillebois, J., Shankle, K., Khanday, I., Mercier, R., Sundaresan, V., & Guiderdoni, E. (2022). High-frequency synthetic apomixis in hybrid rice. *Nature Communications*, *13*(1), 7963. https://doi.org/10.1038/s41467-022-35679-3

*Wang et al—2024—Harnessing clonal gametes in hybrid crops to engineer polyploid genomes*. (n.d.).

Wang, Y., Fuentes, R. R., Van Rengs, W. M. J., Effgen, S., Zaidan, M. W. A. M., Franzen, R., Susanto, T., Fernandes, J. B., Mercier, R., & Underwood, C. J. (2024). Harnessing clonal gametes in hybrid crops to engineer polyploid genomes. *Nature Genetics*, *56*(6), 1075–1079. https://doi.org/10.1038/s41588-024-01750-6

Xu, Y., Jia, H., Tan, C., Wu, X., Deng, X., & Xu, Q. (2022). Apomixis: Genetic basis and controlling genes. *Horticulture Research*, *9*, uhac150. https://doi.org/10.1093/hr/uhac150

# Appendix

A diagram of different colored shapes

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Figure 17. Automatic vs manual pollen diameter measurements. Automatic measurements are in blue, manual are in orange. Number of manual counts is ~50. A shift downwards in pollen diameter can be detected when automatic and manual counts are compared.