Wageningen UR

Controlling meiosis for the benefit of plant breeding

MSc thesis (GEN-80436)

Xiaoqian Shi (880805759080)

Supervisors: Dr. Erik Wijnker and Prof. Dr. Hans de Jong
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Contents

Abstract

Meiosis is a crucial process in plant breeding as this allows breeders to create novel allele combinations. Controlling this process, in terms of crossing over formation and distribution, can effectively influence the outcome of breeding efforts. In classical breeding elite F1 hybrids are created from selected parents. However, selfing such F1s produce offspring in which the superior allele combination will be lost. Here we are exploring a novel technique referred to as Near Reverse Breeding that is helpful in producing parental lines from any heterozygous plant that crossed together will give F1s almost identical to the original material. To this end we used heterozygous *Arabidopsis thaliana* plants for testing different treatments that disrupt second meiotic division in a way that near homozygous, unreduced gametes are produced. Genetic selection of such near homozygous diploid regenerants then allows potential parental combinations for new F1s. The treatments that were tested include high pressure N_2O, high temperature and the spindle inhibitors colchicine and oryzalin. Analyses of pollen morphology showed irregular pollen and strongly varying in size, which were interpreted the result of disrupted meiosis. However, we could not regenerate plants from such pollen, most likely because of timing of the experiments with respect to the stage of microsporogenesis. Another method of modifying meiosis that was tested for potential application in plant breeding makes use of increasing the number of crossing over events. For this purpose Arabidopsis line 1406 was used that was known to display enhanced rate of homologous recombination (HR). Using a visual assay based on fluorescent markers expressed in seeds obtained from a heterozygous plant, we measured meiotic recombination rate in a specific region of one of the chromosome pairs. However, the established HR values between the mutant and wildtype material showed no significant difference.

1. Introduction

1.1 Meiosis and breeding

One of the most important achievements in mankind's history is the domestication of plants and animals for own nutrition that started 20,000 years ago in the Fertile Crescent. The old agricultural system of crops and husbandry became recently an advanced research field of plant and animal breeding that has improved quality and yield needed for providing sufficient food and clothes for our daily life. Breeding technologies aim at obtaining desired characteristics by optimizing their allele combinations. Creating genetic variation here by meiotic recombination is one of the crucial natural processes. There are several essential phases in meiosis: replication of the chromosomal DNA, pairing of homologues, exchanging parts of chromosomes via crossing over, and random assortment of the chromosomes to their daughter cells (Cnudde and Gerats 2005). Diploid cells go through an initial replication of chromosomes and finally result in haploid cells (figure 1). There are two divisions involved which are called meiosis I and meiosis II. Meiosis I leads to reshuffling of allele combinations by intrachromosomal (crossover) and interchromosomal recombinations. Both processes are random events that give rise to countless numbers of new allele combinations. Therefore selection of desired traits in breeding programs is to a greater part dependent on chance.

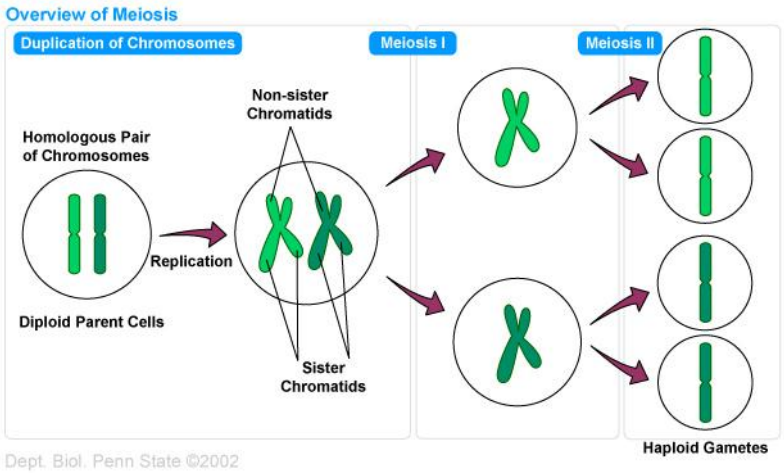


Figure 1: Simplified overview of meiosis (Woodward 2009). The occurrence of crossovers and the production of recombinant chromosomes are left out in this scheme.

As controlled meiotic recombination plays a pivotal role in helping breeders to combine desired alleles (Wijnker and de Jong 2008), the way to do so attracts breeder's attention now more than ever. Several methods have been explored or are being studied on controlling meiotic recombination, such as crossover formation, distributions and frequencies (Drouaud *et al.* 2006; Mezard 2006; Shinohara *et al.* 2008; Dirks *et al.* 2009).

1.2 Heterosis breeding and reverse breeding technique

Genetic improvement of crops has been a slow process until the beginning of the 20th century with the rediscovery of Mendel's laws on heredity by Tschermak, Correns and de Vries (Gerstein *et al.* 2007), after which the knowledge of Mendelian genetics and its benefits for plant breeding accelerated considerably. More recently breeders discovered the phenomenon of growth superiority of hybrids, or heterosis, which became common practice in professional breeding. Heterosis is the phenomenon where a heterozygous F1 from a cross between two inbred parents is more vigorous and has higher yield than either of the two parents. F1 hybrids have also the advantage of bringing together superior characters from both parents in a

way that the hybrid upon selfing will segregate for all these traits and so protects the precious hybrid seeds of the breeder.

Nowadays, breeders heavily rely on F1 hybrid production in modern plant breeding, combined with sophisticated genetic, molecular and statistical methods. Classical plant breeding or forward breeding uses deliberate intercrossing of two homozygous parental lines from several putative parental lines which are already made to produce new varieties (Dirks *et al.* 2003). Subsequently, the combined abilities and effects of new varieties are evaluated after several generations. The best combination and their respective parental line are retained. These parental lines will be used for making F1 hybrids commercially (Dirks *et al.* 2003).

Even though heterosis is bringing a great benefit for breeders, they are still facing a great difficulty in optimizing desired traits due to the unpredictable success of heterosis (Dirks *et al.* 2009). Another difficulty for breeders is generating uncharacterized varieties through seeds, because segregation of trait will result in losing desired allele combinations in the next generation (Dirks *et al.* 2009). Breeders often cross uncharacterized heterozygotes with known inbred lines to evaluate the unknown hybrid. This, however, is notoriously laborious and time consuming.

There is a big challenge for plant breeders to develop methods for preserving desired heterozygotes (Dirks *et al.* 2009). One of the ways to do so is apomixis (Perotti *et al.* 2004), which is asexual formation of seeds without contribution of the paternal gamete. The plants thus formed are genetically identical to their mother plant. Apomixis naturally occurs in more than 400 species, especially in the grass and rosacea families (Bicknell and Koltunow 2004), but their mechanisms and different levels of penetrance can vary enormously between plant groups. In spite of all efforts to understand this “Holy Grail” sufficiently, only few apomictic genes were characterized molecularly so far. In addition, even if such genes are known, their use intrinsically the use of publicly unacceptable transgenic approaches, which makes

the method impossible to apply. Although apomixis has been engineered in *Arabidopsis* (Marimuthu *et al. 2011*), this technique doesn't exist in plant breeding since genetic engineering is rejected by European consumers (Perotti *et al.* 2004). An alternative to apomixis is reverse breeding (RB), which also fix heterozygosity of the F1, but where transgenes needed to silence meiotic crossovers by RNAi are only needed temporarily in the F1 (Wijnker *et al.* 2012). The dihaploid plants thus formed have only partly the transgene, and so, one can easily select for those dihaploids lacking the transgene. Compared to traditional breeding or forward breeding, reverse breeding starts with selecting superior hybrids rather than breeding for homozygous plants which crossed together are expected to produce the F1 combining good traits from both parents (Dirks *et al.* 2003) (Figure 2). In contrast to apomixis, reverse breeding is more flexible in optimizing desirable traits, not just being identical to the parents.

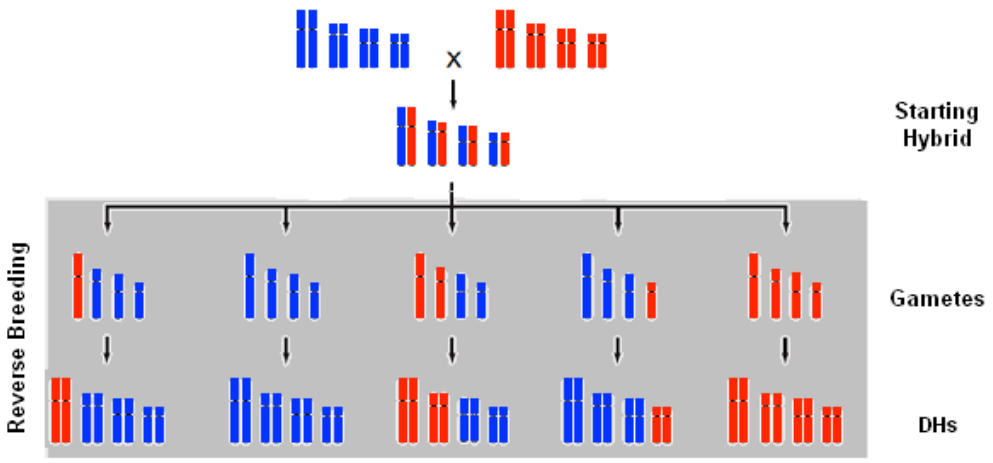


Figure 2: The Reverse Breeding approach, whereby the starting hybrid produces non-recombinant chromosomes. Doubled haploids were produced from these gametes, where after complementing DHs are selected to reconstruct the starting hybrid (Dirks et al. 2003).

Another technique that allows fixation of heterozygotes by constructing complementary homozygote parents is near reverse breeding (NRB). This method is

based on producing unreduced gametes from Second Division Restitution (SDR) (Van Dun and Dirks 2008). SDR can occur, when the cells bypass anaphase II concomitantly with regular crossover events. The resulting plants, which are homozygous in the chromosome part proximal of a crossover, are called SDR-0 plants. Double haploids (DHs) are produced upon marker screening of SDR-0 plants (Van Dun and Dirks 2008). Selection of complementary combinations will now allow reconstruction of new heterozygotes (Dirks *et al.* 2009). Because crossover events still occur during the formation of SDR-0 plants, the new combination may not be completely identical to the starting heterozygote but is highly similar in its genetic composition. Therefore, we call it near reverse breeding (Figure 3).

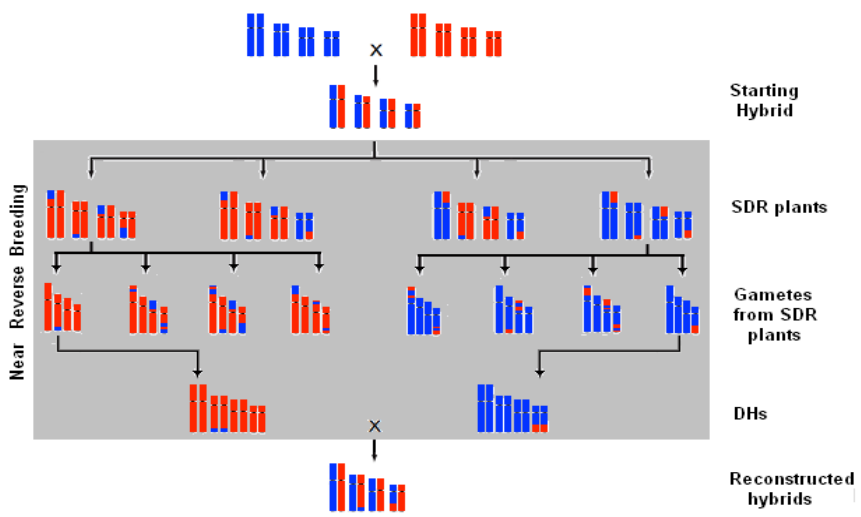


Figure 3: The Near Reverse Breeding approach, whereby SDR gametes / plants are produced of the starting hybrid. Doubled haploids are produced after a haploid induction of the SDRs, so heterozygosity is lost. The goal is to approve to almost complementing homozygous lines, so the starting hybrid is reconstructed (Van Dun and Dirks 2008).

The formation of SDR gametes can be induced by genetic approaches in potato, maize and *Arabidopsis* (Carputo *et al. 2003;* Barrell and Grossniklaus 2005; d'Erfurth *et al.* 2009). Other possibilities to induce SDR formation include the use of chemicals such as nitrous oxide (N_2O) or trifluralin (Okazaki *et al.* 2005; Dewitte *et al.* 2010).

High pressure N_2O gas has been applied during chromosome doubling in some species such as tulips, lilies and begonia, acting as a spindle inhibitor and so to disrupt meiosis in a way that unreduced gametes can be induced (Okazaki *et al.* 2005; Barba-Gonzalez *et al.* 2006; Akutsu *et al.* 2007; Kitamura *et al.* 2009; Dewitte *et al.* 2010). Furthermore, environmental factors, such as temperature, have a great impact on meiosis (Veilleux and Lauer 1981; Negri and Lemmi 1998). Heat shock effects in *Arabidopsis* have been studied and it was found that this in combination with high humidity may result in male sterility during anther development (Kim *et al.* 2001; Warner and Erwin 2005). It has also been reported that one day continuous darkness for *Arabidopsis* also caused male sterility (SMITH and Stitt 2007). Moreover, diploid gamete can be induced under a high temperature environment in *Rosa* (Pécrix *et al.* 2011).

1.3 Meiotic and somatic HR in plants

Homologous recombination (HR) is a vital cellular process which happens between homologous chromosomes, involved in genome maintenance processes such as DNA repair, replication, telomere maintenance, and meiotic chromosomal segregation (Lieberman-Lazarovich and Levy 2011). In sexual eukaryotes including most plants, there are two types of homologous recombination (HR), meiotic HR and somatic HR. However, somatic HR is rare events in plants compared to that in animals (Bleuyard *et al.* 2006). Instead plants use a pathway, which allows them to repair DNA damage via non-homologous end joining (NHEJ). In this respect plants do differ in meiotic HR from animals. All chromosomes have at least one crossover, and in general 1-2 per chromosome arm (Lieberman-Lazarovich and Levy 2011). Higher crossovers would be very interesting for breeders as it may be used for obtaining novel allele combinations between closely linked genes or in chromosome regions where crossovers are suppressed (Wijnker and de Jong 2008). However the mechanism that regulates the number of crossover is unknown. There are some artificial methods to

induce the crossover, such as applying chemicals, heat shock and UV (Wijnker and de Jong 2008). Moreover, overexpress or silence of genes which are regulating recombination can also lead to enhancements of crossover rates. Crossing over events are highly depended on the rate of non-sister chromosome (homologous) recombination during meiosis, and also meiotic HR shares similarity with somatic HR in the way of occurring between homologue chromosomes. Therefore, increasing the HR rate by chemical treatment of mutation, resulting in higher numbers of meiotic crossovers, would be desirable. Here, we propose to test the hypothesis that increasing somatic homologous recombination may also elevate the rate of such processes at meiotic prophase I (HR).

In recent years, an Arabidopsis line 1406 was constructed with increased rate of somatic HR (Gherbi et al. 2001). This line was generated by using zinc finger artificial transcription factors (ZF-ATF) which consist of polydactyl zinc finger (PZF) DNA binding domain linked to a protein domain that activate or suppress gene expression (Lindhout *et al.* 2006). Zinc fingers (ZF) can bind to three contiguous DNA bases and so ZF-ATF were constructed to recognize unique sites within a complex genome, such as GNN, ANN (N is for any of the four nucleotide). Based on this, the ZF-ATF method was used to screen for phenotypes as it creates random mutagenesis. Through application of a ZF-ATF containing a 9 bp DNA recognition site, HR in *Arabidopisis* was unregulated. In the *Arabidopsis* line 1406, containing the aforementioned ZF-ATF, an interrupted GUS reporter gene, which can be restored after HR events, has been inserted (Swoboda *et al.* 1994). Individual recombination can be observed as blue spots or sectors via GUS staining (Lindhout *et al.* 2006). It has been illustrated that this 1406 line led to 200-1000 fold increase in somatic HR (Lindhout *et al.* 2006). To monitor the HR frequency in Arabidopsis (Gherbi *et al.* 2001), in this study this target line was used as a pilot.

Morphological markers are very useful in monitoring recombination rates, as they can be directly being phenotyped compared to DNA markers that need molecular analysis. For this purpose, an *Arabidopsis* test line will be used, containing a pair of genetic markers linked in *cis*, namely GFP and RFP, which can be scored in seeds, because the gene inserted behind a seed specific promoter (Melamed-Bessudo et al. 2005). This mutant is ideal for testing the rate of crossing over by counting the number of different colors of fluorescing seeds.

In this study, we are aiming at exploring two ways of modifying meiosis, near reverse breeding by inducing SDR gametes formation and increasing crossover frequencies. If we can generate SDR gamete in order to generate SDR plants? If SDR plants can be generated, will near reverse breeding be the promising way to fix the heterozygosity? Furthermore we test *Arabidopsis* line 1406, aiming to find out if the construct with increased HR can increase crossover events in *Arabidopsis*.

2. Material and methods:

2.1 *Arabidopsis thaliana* accessions:

Columbia (Col), Landsberg *erecta* (L*er*), Col X L*er* F1 hybrid, male sterile L*er*, and the CenH3 haploid inducer were used in experiments of inducing SDR gamete.

Arabidopsis line, 1406 with increased HR and Arabidopsis test line (was kindly offered by Dr. Beatrice L. Lindhout, Leiden University) and Col3-4/20 (Melamed‐Bessudo *et al.* 2005) were used for estimating meiotic recombination rates. Col3-4/20 contains red (RFP) and green (GFP) fluorescent protein genes in the Columbia background and at a genetic distance of 25 cM (Melamed‐Bessudo et al., 2005).

2.2 Crossings:

The following crossings were made:

1. F1 hybrid from a cross between Col and L*er*.
2. CENH3 (maternal) crossed with F1 (Col X L*er*) treated with N_2O or High temperature.
3. A male sterile Landsberg *erecta* (L*er*) crossed with F1 (Col X L*er*) treated with N_2O or High temperature (42 °C).
4. A male sterile Landsberg *erecta* (L*er*) was pollinated with the 1406 line. The resulting F1 was back-crossed with male sterile L*er*. The F1s were also selfed. The resulting BC1 and F2 were studied under the fluorescence microscope. Figure 4 shows the genotypes of the parents and F1, and the BC1 and F2 progenies.

2.3 Fluorescence microscopy

Green (GFP)/ Red (RFP) fluorescent protein in the mature dried seeds was studied under a fluorescent Zeiss Axioskop microscope equipped with GFP and RFP filters.

2.4 Seed germination

Arabidopsis seeds were placed on a piece of moistened filter paper in a 51 x 15 mm Petri dish sealed with Parafilm. Seeds were incubated at 4 °C to break dormancy and to ensure synchronization of the germination for the duration of 7 days. The Petri dish with the seeds was kept in a climate chamber (photoperiod 16L: 8D at 20°C) for two days. For germination we transferred the seeds with a wet hairbrush to a 51-wells tray (30 x 50 cm) containing soil. The seeds were kept in the climate chamber (photoperiod 16L: 8D at 20°C) for germination and the plants were kept there till flowering.

2.5 N_2O treatment:

N_2O treatment was performed in a pressure tolerant vessel at 6 bars for the duration of 15 hours, 24 hours, 36 hours and 48 hours, respectively. Compression time took 1.5 hours, while decompression was more than 3 hours to avoid physiological damage of the plant. After treatment, plants were grown in the greenhouse for 7 days and then used for further analyses.

2.6 Heat treatment:

Plants (Col X L*er* hybrids) were placed in an incubator with light on (4000 lux). Temperature was increased gradually from room temperature (about 24 degree) to 42 ℃ in 40 minutes. Then the plants were kept in 42 ℃ for 3 hours. After 3 hours the temperature was decreased slowly from 42 ℃ back to room temperature. After treatment, plants were kept in the greenhouse.

2.7 Chemical treatments

Colchicine treatment

Open flowers of Arabidopsis (L*er* X Col) were immersed into the Colchicine solution for 2 hours, and the flowers were kept up-side down. Colchicine solutions of 0.1% and 0.3% in 0.03% Silwet-77, 0.1% DMSO and 3% MS buffer were used. The Silwet-77 and DMSO reagents helped to enlarge the contact area of flower bud and facilitate the flower bud to absorb chemicals.

Oryzalin treatment

The Oryzalin stock solution was kindly given by Dr. Jaap van Tuyl, Laboratory of Plant Breeding. We diluted the stock to working solutions of 0.001% and 0.003%. The stock solution was slowly added to the water to prevent crystallization and

immediately used on the day of the treatment. Open flowers of Arabidopsis (L*er* X Col) were subsequently immersed in the oryzalin solution for 2 hours or 3 hours. After the treatment, the flower buds were rinsed in milli-Q water in order to wash off the chemical residue.

2.8 Pollen morphology analyses

After the different treatments we kept the plants in the climate room. The flower buds of the Col, L*er*, cvi, F1 plants from the N_2O treatment was checked after 6, 7 and 8 days; those of the F1 from the heat treatment were checked after 7 and 8 days. For staining the pollen we transferred one anther from the new opened flower to a drop of lactophenol funchsin on a slide covered by an 18mm X 18mm cover slip. Pollen grains were studied in a Zeiss AxioPlan bright field microscope using 25x and 40x optics. Images were captured with a CCD camera for pollen measurements using the Image-J software (http://rsbweb.nih.gov/ij/) together with pollen plugin program (from Sylvester de Nooyer, Rijk Zwaan Inc.).

2.9 Cytogenetic analyses

The flower buds were fixed directly after heat treatment with a freshly prepared Carnoy fixation solution (3x Absolutes ethanol : 1x acetic acid) for 24 hours at 4 °C. Fixed flower buds were transferred to a 70% ethanol solution and kept at 4°C.

After fixation of flower buds in Carnoy solution, buds were rinsed three times with Mili-Q water for 5 minutes per each time. Then the buds were transferred to 10 mM Na citrate buffer (pH=4.5) and kept for 5 minutes. Anthers were washed three times in milli-Q water and 1x Na-citrate buffer (pH 4.5), and then incubated in an pectolytic enzyme mix (1:10 of 1% enzyme stock in the citrate buffer), in the same buffer at 37°C for 2 hours. The duration of the enzyme treatment may be longer or shorter depended on the enzyme activity. After enzyme treatment the material was rinsed in buffer and milli-Q water and kept at 0-4°C until further use. Cell spreads were made

by transferring a single bud onto the middle of a clean and dry slide, dissect the material with a fine needle in a small drop of 45% acetic acid and tapped gently to release pollen mother cells. Then the cells were stirred gently and spread over a small circle area by the needle on a hotplate at 45°C for 2 minutes. During the spreading, the needle was barely touching the glass slide, as the touching would damage the cells. Finally, the slides were stained with 15 µL DAPI / Vectashield solution after 30 min baking in 65°C oven. Then each slide was covered with a 24mm x 50 mm coverslip and squashed between filter paper to remove excess stain solution. The slides were studied under a Zeiss Axioplan II imaging photomicroscope (http://www.zeiss.com/) equipped with epifluorescence illumination and small band filter for DAPI fluorescence. Selected images were captured using a Photometrics Sensys 1305 · 1024 pixel CCD camera (Photometrics, http://www.photomet.com).

2.10 Pollen staining with DAPI

The anther was placed on amslide with 12 µL of DAPI working solution which is mixture of 2.5 µg/mL DAPI, 0.01 % of tween, 5% of DMSO, 50 mM PBS (pH 7.2). The slide was coved with a covering glass and placed in the dark until the rim of the covering glass is dry (about 30 minutes), then sealed with nail polish. Incubation of the slide was taken place in the dark at 4°C for 4-12 hours. Slides were checked under a fluorescence microscope with DAPI filter.

2.11 SNP analysis and sequencing

The genotypes of the *Arabidopsis* plants were established with control of the starting material, recombination patterns and SDR-0 plants could be observed by SNP analysis. For this analysis, *Arabidopsis* plants leaves were placed in a 96 wells plate containing 100 µL of lysis buffer in each well. The prepared plant materials were genotyped by Bastiaan de Snoo of Rijk Zwaan Breeding Campany, Fijnaart, the

Netherlands. Plant materials were tested for 36 SNP markers equally distributed over the 5 chromosomes (depicted below).

A

	Chromosome 1									Chromosome 2						Chromosome 3							Chromosome 4						Chromosome 5						
	592939	3504562	wak1	M235	AIGI	GAPB_2	19996564	M305	ADH	29393153	172469	3994520	7994335	11991100	15493536	18753024	580137	4000301	8000279	13491841	15990167	20428680	23443472	641363	4040382	8001301	11999130	14177230	18488307	342420	RCL1B	8000694	11982076	FM4_2	23115566	26479586
	B8	G2	B7	A8	A2	A7	G3	A9	A1	B11	C1	G4	G5	G6	G7	C4	C6	G8	G9	G10	G11	C11	G12	C12	H1	H2	H3	D4	H4	D6	B1	H5	H6	A4	D9	H7
Columbia	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Landsberg erecta	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

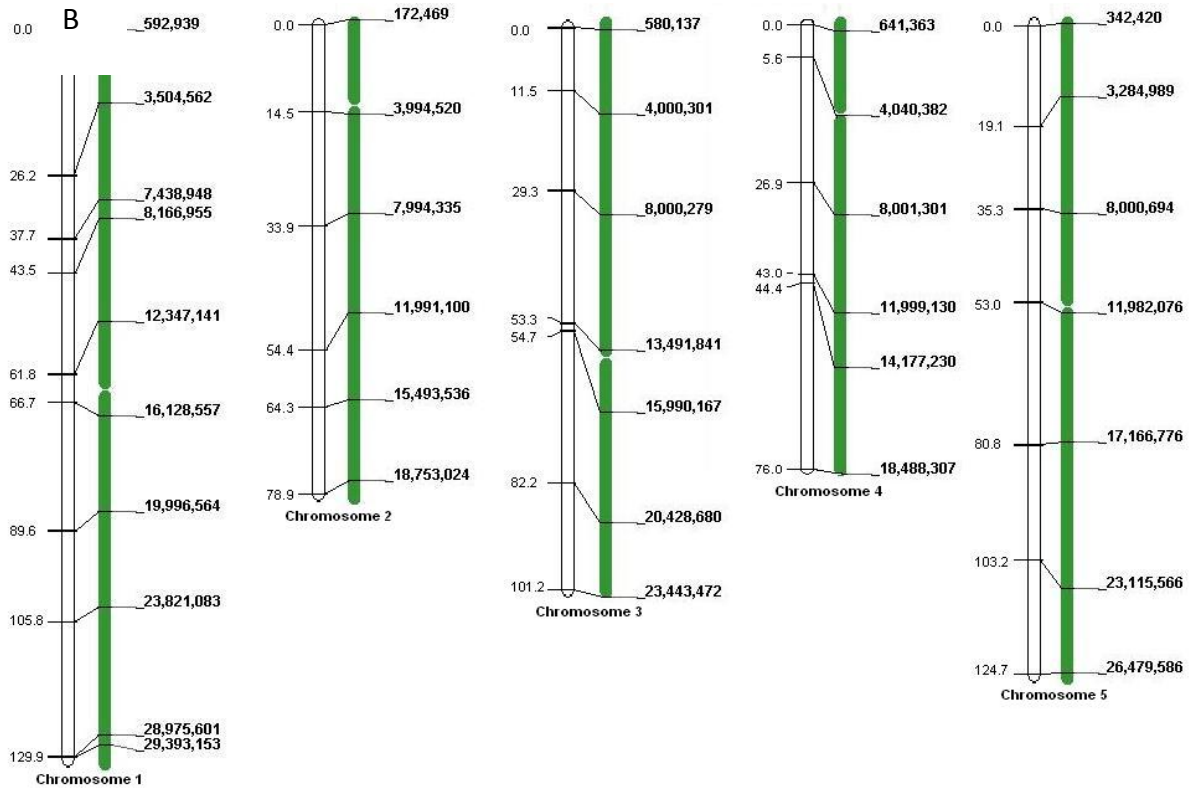


Figure 4: SNP analysis results of the two parental plants. In total 36 SNP markers were used. Letters are marked in black are (near-) centromere makers. Blue and pink are markers on long and short arms respectively (A). The genetic map (white bars) of *Arabidopsis thaliana*, based on the recombination pattern of F1 hybrid, Columbia and Landsberg erecta (Joinmap 4). The marker positions are expressed in cM. Besides the physical map is shown (green bar) where the marker position is expressed in base pairs. (B)

2.12 Plant stress

Darkness experiment was performed by covering the heterozygous plants (L*er* X Col) with boxes for 24 hours, 48 hours. The darkness treatment started from 8:00 am, 12:00 and 17:00 pm in the afternoon. During the dryness experiment, the heterozygous (L*er* X Col) plants were treated 3 hours at 42 °C, without watering one day before the treatment.

3. Results

3.1 Changes of pollen morphology after N_2O gas and heat treatments

We used the Col and Cvi accessions for the effect of N_2O treatment for the duration of 24, 39 and 48 hours. We performed each experiment for the given duration twice. Pollen morphology was checked 6, 7 and 8 days after the treatment. Both 24 hours treatments resulted in regular sized pollen. In one of the two experiments with duration of 39 hours, plants died in a few days after treatment. Irregular sized pollen were observed 8 days after the another 39 hours treatment (Figure 5 A, B). 48 hours (twice) experiments resulted in dead plants few days later.

We treated F1 plants (Col X Ler) for 24 and 36 hours, respectively. Only the plants of the24 hours experiment showed irregular sized pollen. The heat treatment (42℃ for three hours) resulted in irregular pollen as well.

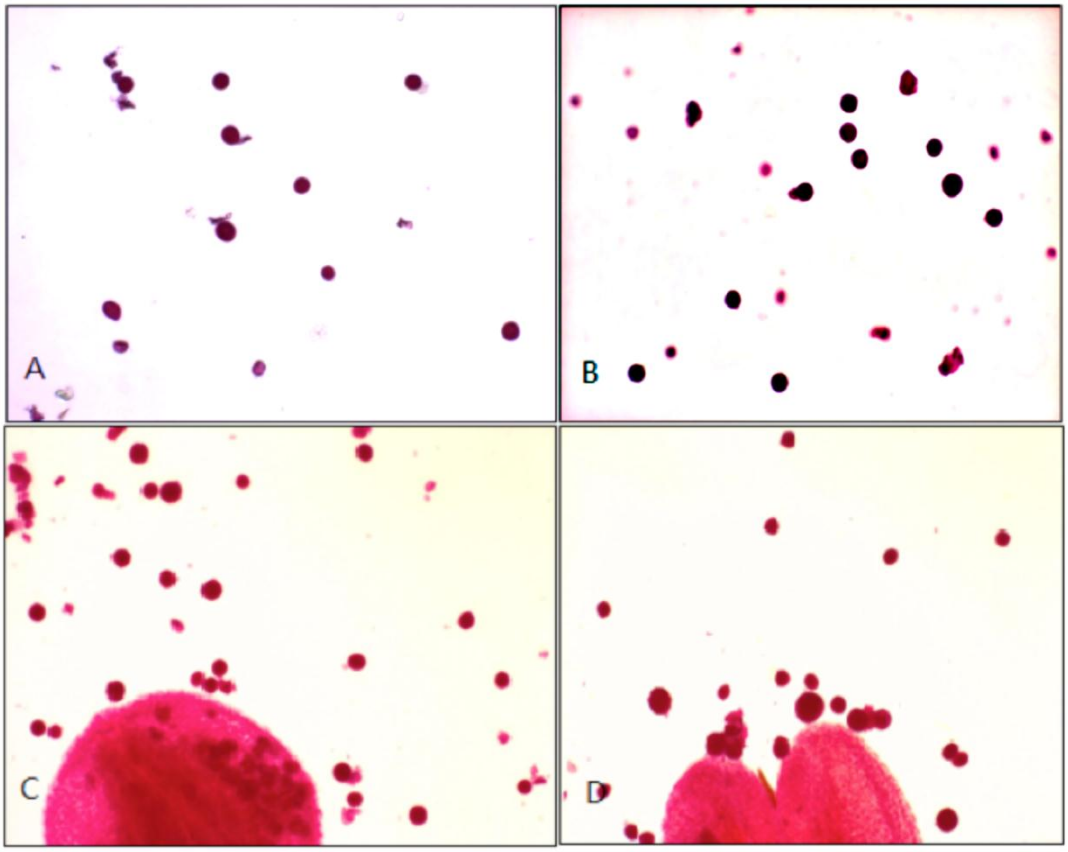


Figure 5: Pollen morphology from samples obtained after the N_2O and heat treatments (photographs are from random samples in the pollen grain slides. (A) and (B) are pollen from *Arabidopsis* Columbia and treated with N_2O for 39 hours. (C) is pollen from heterozygous *Arabidopsis* crossed between Columbia and Landsberg *erecta*, treated with N_2O for 24 hours. (D) Heterozygous *Arabidopsis* pollen treated at 42℃ for three hours.

Pollen samples were photographed and their size quantified with the pollen plugin of ImageJ. As shown in Figure 7, different pollen size distributions were observed between treated (with N_2O) and non-treated pollen (wild type). The red bars indicate the pollen size distribution of untreated (wild type) Col ecotype. Two major peaks were observed for treated pollen (blue bars), one about the same size as the wild type and another peak at bigger pollen sizes.

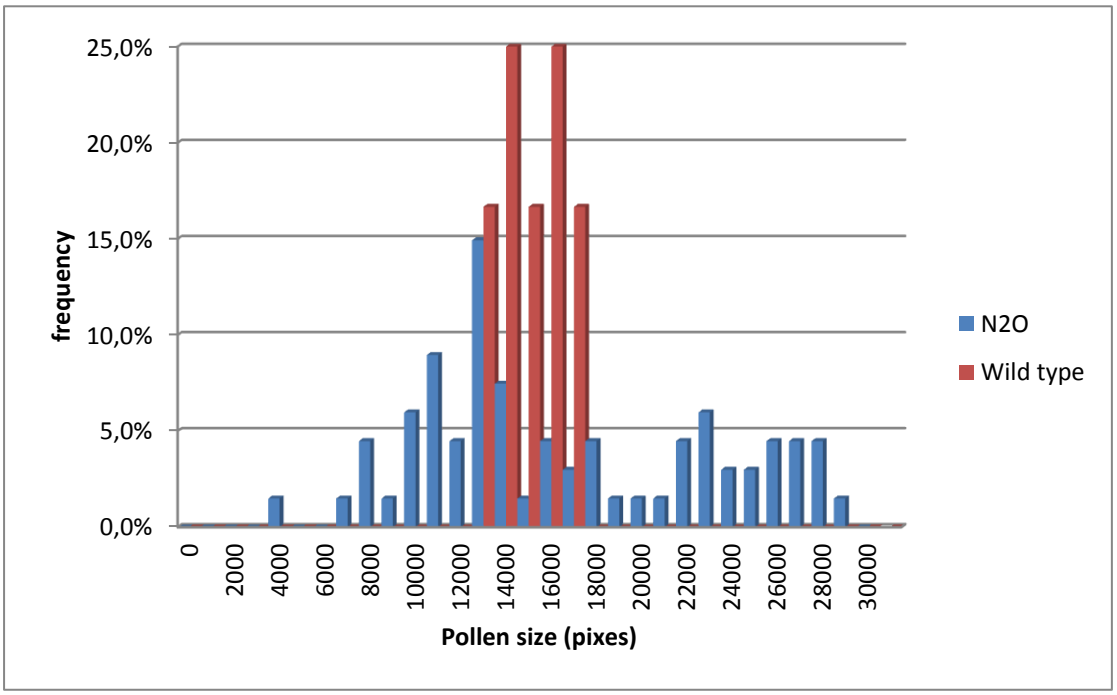


Figure 6: Distribution of pollen size in *Arabidopsis* for Columbia ecotype. Blue bars stand for N_2O treatment for 39 hours. Red bars are for untreated (wild type) pollen.

The distribution of pollen size was established by the measurement of the pollen plugin as previously described (in Materials and Methods). Pollen can form different of distribution profiles based on ploidy level. In arabidopsis, pollen from diploid plant (haploid pollen) forms a single peak distribution (as the Figure 6 show for the wild type pollen). Unreduced/diploid pollen produce a bimodal pollen size distribution, with the low and the high value peaks corresponding to the sizes of haploid and diploid pollen grains, respectively. Different from unreduced/diploid pollen sized distribution, higher ploidy level of pollen size distribution produced many small peaks which are corresponding to triploid or tetraploid pollen gain. In the Figure 7 it is shown the different pollen size distributions are caused by N_2O treatment and heat treatment. The green bars indicate pollen size distribution from the heat treatment, which form three peaks. The third peak of heat treatment indicated pollen is with higher ploidy level than diploid pollen.

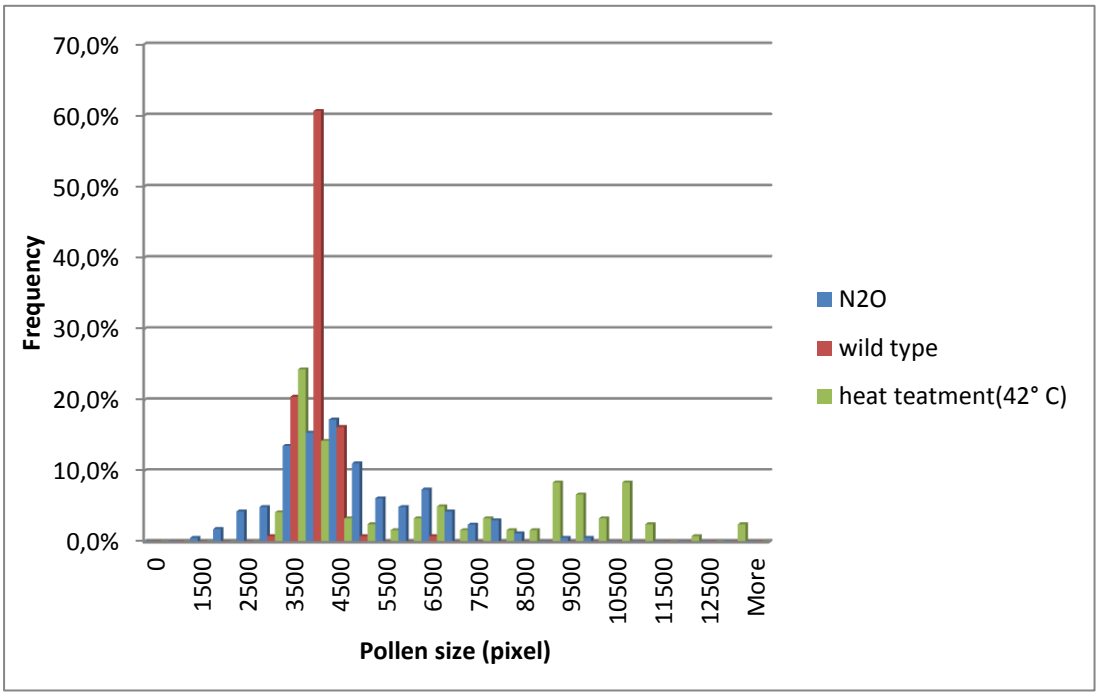
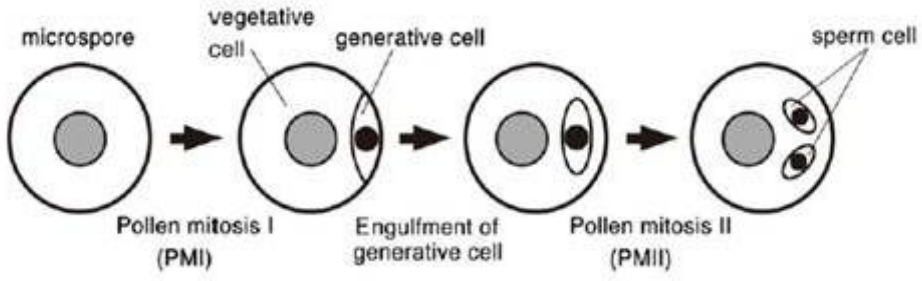


Figure 7: Distribution of pollen grain in Arabidopsis F1 hybrids (cross between Colombia and Landsberg *erecta*) established for heat treatment at 42 °C, N_2O treatment at 6 bar for 24 hours and untreated.

3.2 pollen staining with DAPI

To validate the larger pollen grains, nuclear DNA staining was performed by staining with DAPI. In *arabidopsis*, mature pollen is formed after mitosis (Figure 8Ⅰ). Asymmetric division of uninuleate microspores at first pollen mitosis produces binuleate pollen of a vegetative nucleus and a generative nucleus. Subsequently, division of the generative nuclei produces a pair of sperm cell forming trinucleate pollen (Matsushima *et al.* 2011). Larger pollen grains (pointed by blue arrows) clearly showed enlarged nuclei, indicating high DNA content of these bigger pollen gains (Figure 8 II).

I



II

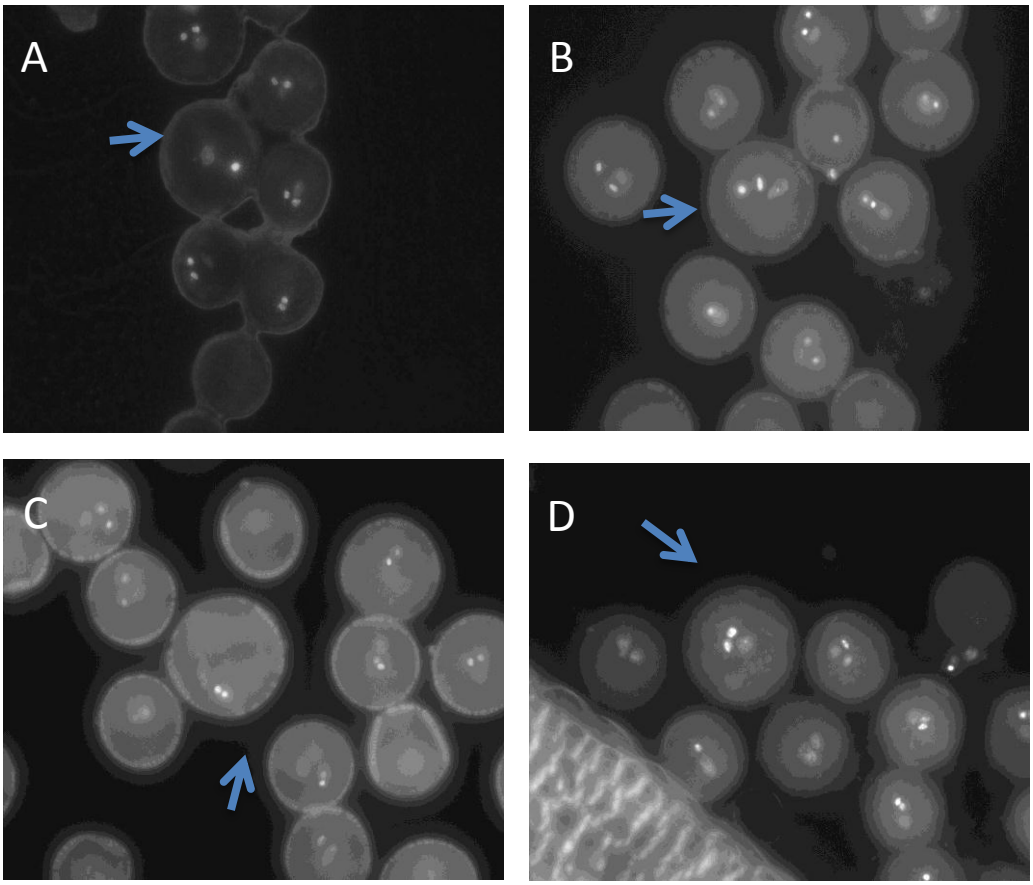
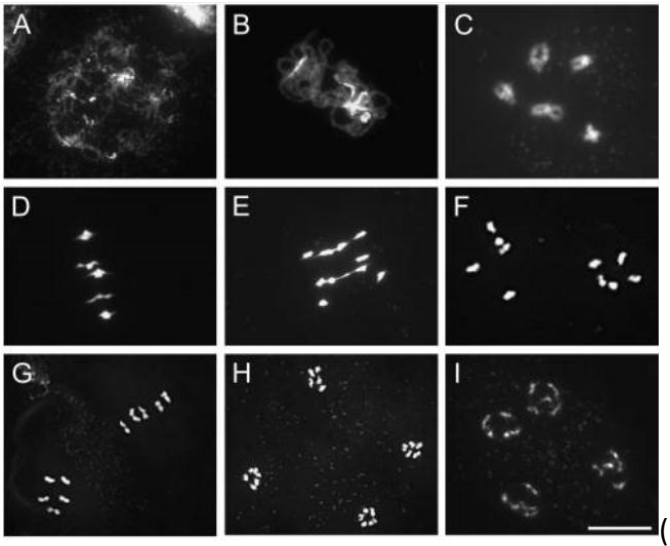


Figure 8-I: Pollen maturing process in arabidopsis (Matsushima et al. 2011). II: Pollen morphology as revealed by DAPI staining. The pollen was treated with N_2O after 7 days. The two brightly florescent nuclei in the trinucleate pollen grains correspond to generative nuclei; the other less condensed nucleus is vegetative nucleus nucleus.

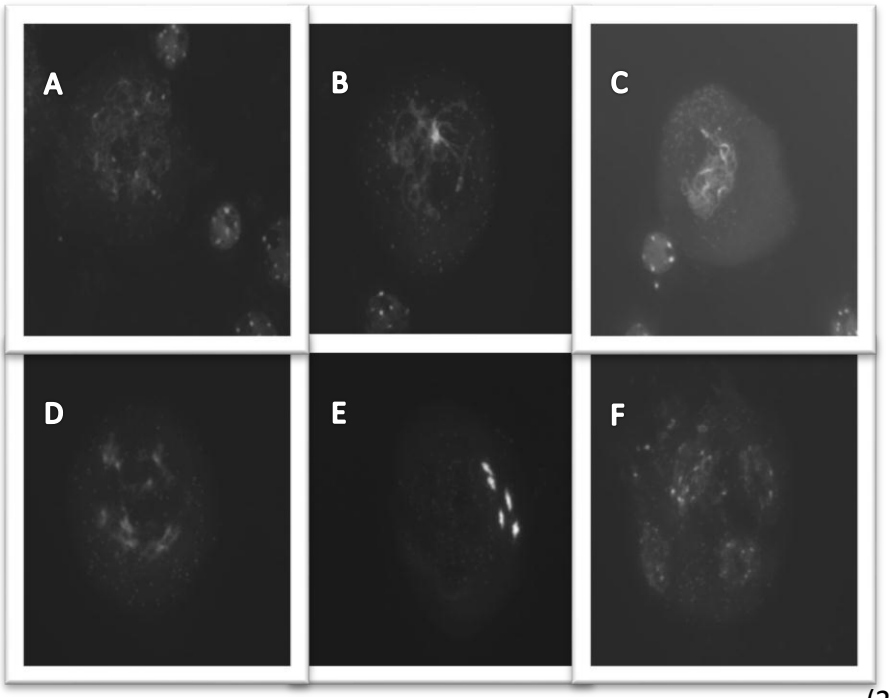
3.3 Cytogenetically analysis of heat treated meiosis

To confirm that high temperature interrupts certain stages of meiosis, meiotic chromosome behaviour was investigated using chromosome spreading technique. During our observation, meiosis I progressed normally and indistinguishably from

the wild type (adapted from De Storme & Geelen, 2011). Following chromosome condensation and crossover formation in prophase (Figure 9 (1) A-C (2) A-C), the metaphase spindle aligns the five bivalents with their chiasmata to the equatorial plane (Figure 9 (2) D). This phase could not be captured in our experiment. Subsequently, the homologs segregate towards two poles at anaphase (1) E (2) D-E, leading to two polar sets of five chromosomes at the end of meiosis. I. If the heat treatment affects the meiosis resulting in tetraploid pollen, we would expect that there are two diploid or one tetraploid nucleus rather than four haploid nuclei at late stage of meiosis (tetrad stage). However, no abnormal meiosis events were observed.



(1)



(2)

Figure 9: (1) Meiotic chromosome behaviour of wild type *Arabidopsis* (Col-0) (De Storme and Geelen 2011). (2) Cytological analysis of meiosis in *Arabidopsis* treated with high temperature (42℃). (A) Early prophase I stage, chromosomes begin to condense. (B) Pachytene stage. (C) Diplotene stage. (D) Diakinesis stage with five bivalents in which homologous chromosomes are attached to each other by chiasmata. (E) Anaphase I showing chromosome on separated metaphase plates. (F) Tetrad stage before cytokinesis containing four haploid nuclei.

3.4 Genotyping data

In order to test whether the larger pollen in *Arabidopsis* contains 2n SDR gametes, we attempted to cross 2n pollen with the CENH3 inducer line. However, not all the crosses between 2n pollen and haploid inducer line, CENH3, lead to fully grown seeds. More than 50 % of the seeds were smaller, irregular and darkly stained and often shrivelled (Figure 10). We also transferred 2n pollen onto the virgin pistils of male sterile Landsberg *erecta*. The seeds obtained from this cross were supposed to be triploid seeds (3X), which are bigger than normal diploid wild type seeds (2X). However, seeds that we obtained from this cross are very regular in size and no bigger (triploid) seeds were observed.

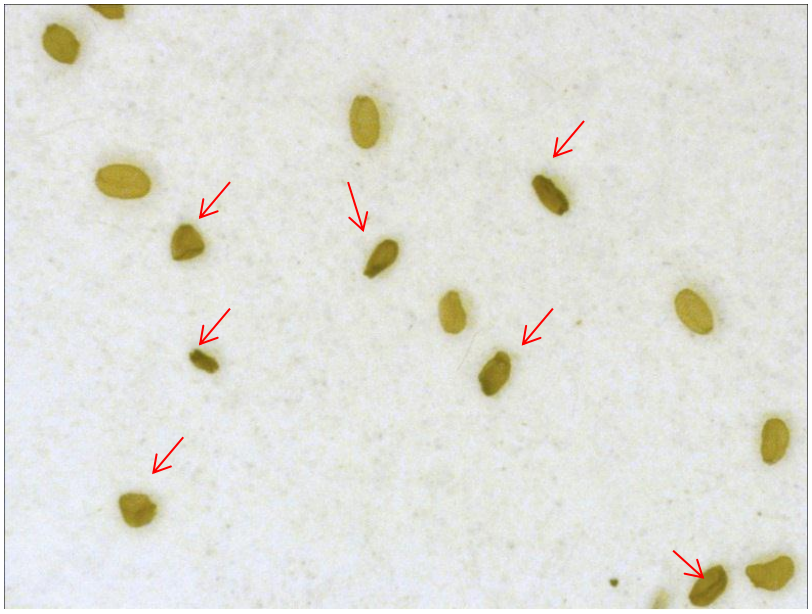


Figure 10: seeds which are crossed between 2n pollen and CENH3 inducer line. Bigger and round seeds are able to germinate into plants. Smaller and irregular sized seeds are shrivelled and failed to germinate into plants (indicated by red arrows).

N_2O treatment

2n pollen induced from N_2O treatment was crossed with CENH3 haploid inducer line. Genotypes of offspring were tested by centromere marker (GAPB_2, 3994520, 13491841, 4040382 and 11982076). “B” letters (in blue) stand for plant with

Landsberg genotypes. “A” letters (not presented here) stands for Col genotypes. “H” is heterozygous for “A” / “B”. From the genotyping data, the plants not SDR plants as SDR plants should be homozygous at centromere region.

		GAPB_2	3994520	13491841	4040382	11982076
		A7	G4	G10	H1	H6
A1	1	H	H	B	B	B
A2	2	B	B	H	B	H
A3	3	H	B	B	H	B
A4	4	B	H	H	B	B
A5	5	B	H	B	H	B
A6	6	B	H	B	H	H
A7	7	B	H	H	H	B
A8	8	H	H	B	H	H
A9	9	H	B	B	H	B
A10	10	B	H	H	B	H
A11	11	B	H	B	B	H
A12	12	H	B	B	B	B
B1	13	H	H	H	H	H
B2	14	B	H	H	B	H
B3	15	H	H	H	B	H
B4	16	H	B	B	H	B
B5	17	H	B	B	B	H
B6	18	H	B	B	B	H
B7	19	B	B	B	B	H
B8	20	H	B	H	B	B
B9	21	B	H	H	B	H
B10	22	H	B	B	H	B
B11	23	B	H	B	H	B
B12	24	H	H	H	H	H

Figure 11: Genotypes of plants obtained from cross between heterozygous plant (Col X Ler) treated with N_2O and CENH3. The A7 (GAPB_2), G4 (3994520), G10 (13491841), H1 (4040382), and H6 (11982076) are (near to) centromeres markers. “B” letters (in blue) stand for plant with Landsberg genotypes. “A” letters (not presented here) stands for Col genotypes. “H” is heterozygous between “A” and “B”

Heat treatment

The 2n pollen that was induced by heat treatment was pollinated on the CENH3 inducer line. Offspring from the cross are genotyped. The genotyping data can be seen in Figure 12. From the genotyping data we concluded that no SDR-0 plants are formed. Putative SDR-0 plants should have genotypes that are homozygous at the regions that are proximal of the crossover. Most of the plants are heterozygous in

this region, for example: plants D11, E12, G8, B3, B4, C7, and E7. Although all other plants are homozygous there, most of them are more likely haploids. Some of the plants, such as C3, D7 and H2, have one or two markers, which indicate heterozygosity for that maker. In the plant B4 and E7, both chromosomes 1 have heterozygous sections at the distal end and chromosomes 4 are heterozygous. This is likely because of chromosome translocation from chromosome 1 to chromosome 4.

tabel 2	592939	3504562	wak1	M235	AtGi	GAPB_2	19996564	M305	ADH	2939153	172469	3994520	7994335	11991100	15493536	18753024	580137	4000301	8000279	13491841	15990167	20428680	23443472	641363	4040382	3001301	11999130	14177230	18488307	342420	RCL1B	8000694	11983076	FM4_2	23115566	26479586
	B8	G2	B7	A8	A2	A7	G3	A9	A1	B11	C1	G4	G5	G6	G7	C4	C6	G8	G9	G10	G11	C11	G12	C12	H1	H2	H3	D4	H4	D6	B1	H5	H6	A4	D9	H7
C3	A		B	B	B	B	B	B	H	H	A	A	A	A	A	A	B	B	B	B	A	A	A	A	A	H		H	B	A	A	B	B	B	A	A
D7	B	B	B	B	A	A	A	A	A	A	B	A	A	A	A		H	A	A	B	B	B	B	A	A	A	A	A	A	B	B	A	A	B	B	B
D11	A		A	B	B	B	B	B	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	H	H	H	H	H	B	B	B	B	A	B	B	B
E12	B	B	B	B	H	H	H	H	H	H	B	B	B	H	H	H		H	H	A	A	A	A	B	B	H	A	H	H	H	H	H	H	B	B	H
F3	B			B	A														A	A		B			A			A							B		
G2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	A	A	A	A	A	A	A	A	A	A	H	B	B	A	A	A	B
G4	B	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	H	A	A	A	A	B	B	B	B	B	B	A	A
G8			H	H	H	H	H	H	B	B	B	B	A	A	A	B	A	A	A	A	B	B	B	B	B	B	B	B	H	B	B	B	B	B	A	A
H2	A	A	A	A	A	A	A	A	H	H	A	A	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A3	A	A	A	A	A	A	B	B	B	B	A	A	A	A	A	B	H	A	A	B	B	B	B	A	A	B	B	B	A		A	A	A	A	A	A
B3	A	A	A	B	B	B	B	B	A	A	B	B	B	A	A	A	A	A	A	A	A	B	B	H	H	H	H	H	A	B	B	B	B	B	B	A
B4	B	A	A	A	A	A	A	A	H	H	A	B	B	A	A	A	A	A	B	B	B	B	B	H	H	H	H	H	A	A	A	A	A	A	B	B
C7	A	A	A	B	B	H	A	A	A	A	A	A	A	A	A	B	A	A	B	B	B	B	B	A	A	A	B	B	B	A	A	A	A	A	A	A
E7	A	B	B	B	B	B	B	B	H	H	A	A	A	A	A	A	B	B	A	A	A	A	A	H	H	H	H	H	A	A	B	B	B	B	A	A
Col	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
R29	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

Figure 12: Genotypes of plants obtained from cross between heterozygous plant (Col X Ler) treated with high temperature and CENH3. Letters in red refer to the markers closest to the centromere. "A" (marked in red) is with Colombia background and "B" (in blue) is with L*er* background. "H" is heterozygous between "A" and "B"

3.5 Plant stress

Since a lot of unexpected factors are affecting meiosis or plant development during N_2O treatment or heat treatment, 2n pollen is difficult to obtain constantly. In two pilot experiments, we tested the effect of darkness and dryness on the performance of pollen size.

Darkness

The heterozygous (Ler X Col) wild type plants were placed under darkness for 24 hours, 48 hours. The darkness treatment stared from 8:00 am, 12:00 and 17:00 pm in the afternoon. The plants were checked seven days after the treatments. All plants formed siliques filled with good viable seeds, except for the plants obtained from the 48 hours darkness treatment. Normally, siliques are formed after

fertilization takes place. The 48 hours treatment leads to abortion of very young seeds in short siliques. This may because the abnormal light cycle massed up male or female meiosis, or led to fail of fertilization.

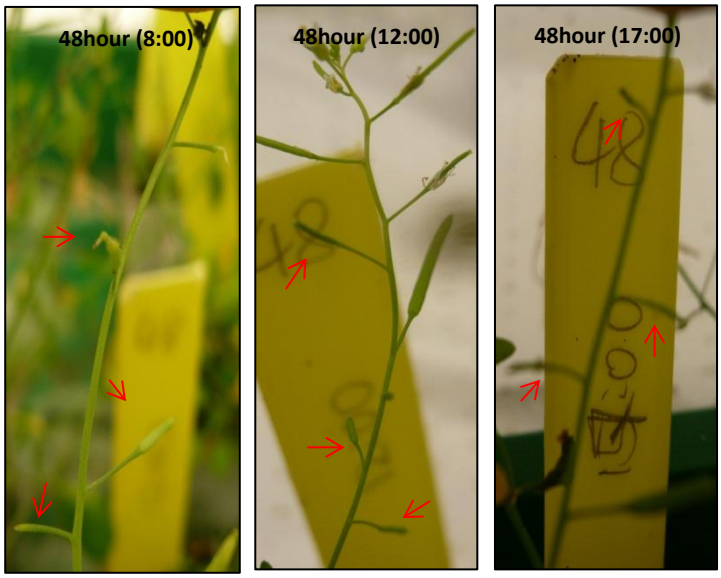


Figure 13: effects of darkness treatment after 7days. The darness treatment lasted 48 hours and started at 8:00, 12:00 and 17:00 respectively. Red arrows indicate abnormal developed siliques.

Dryness

The heterozygous (Ler X Col) plants were treated as in the heat treatment (3 hours at 42 °C), except without watering one day before the treatment. The plants were checked after 7 days, which is illustrated in Figure 14. This treatment led to abortion of flowers, leaving a bare segment on the floral stem.The floral meristem survived, allowing the formation of new flowers. Together with high temperature, dryness would interact with other signals to influence developmental events, which resulted in flower abortion.

Figure 14: Response of the floral shoot of *Arabidopsis thaliana* to heat treatment without watering for one day before the treatment.

The photograph was taken one week later. Flowers aborted after exposure of high temperature treatment without one day watering.

3.6 induction of 2n pollen by other chemicals

In order to obtain 2n gametes more efficiently, other treatments were performed. Colchicine and Oryzalin were used to stop second meiotic division. Unfortunately, there was no 2n pollen obtained.

The Colchicine is used for chromosome doubling in Brassica. It has been reported that colchicine induced autotetraploid in *Arabidopsis* (Wilna ecotype) (Weiss and Maluszynska 2001). We wanted to try if colchicine can also lead to unreduced gametes in *Arabidopsis*. The same concentration which has been used for Brassica chromosome doubling was performed. Three or four days after treatment, the plants died. We concluded that DMSO might be the reason for the plant death as it is toxic to plant and animal cells (Brayton 1986). Therefore, lower concentration of DMSO was used. Although the treatment did not lead to plant death, there was no 2n pollen observed.

Oryzalin is a well-known chemical to induce 2n pollen for increasing the ploidy level. For Arabidopsis, it has been studied that oryzalin has effects on microtubules (Nakamura et al., 2004; Paredez et al., 2008). In out experiment, 7 days after the treatments, it did not show any variation in pollen size.

3.7 Meiotic recombination frequency analysis

A GFP/RFP tester line was used to establish crossover recombination between the loci for these two linked colour markers (genetic distance is 20 cM). This line was crossed with plants showing a higher somatic homologous recombination rate. Seeds resulting from this cross and containing the linked GFP and RFP markers were

crossed with male sterile landsberg *erecta* (L*er*) in order to generate BC1. The seeds were also generated into F2 by self-pollination. (See in the figure 15 a)

In BC1 seeds, GFP/RFP marker is heterozygous as figure shown below (Melamed‐Bessudo *et al.* 2005).

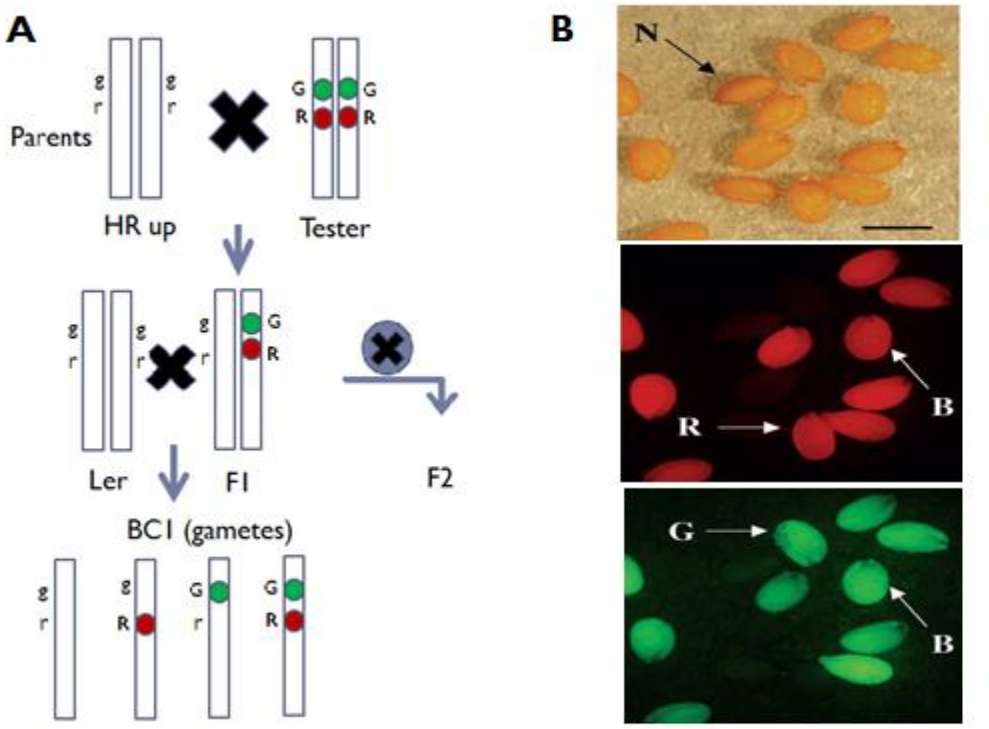


Figure 15: A seed-based fluoresenct assay to estimatie meiotic recombination

A: crossing scheme for measuring crossover recombination rate. Plants with higher somatic homologue recombination rate (HR up) crossed with GFP/RFP tester line. BC1 and F2 were used to measure the recombination rate

B: Visualization of *Arabidopsis* seeds under visible light (first photo) and two different filters, one for RFP (middle) and one for GFP (bottom). In the photos, R stands for only RFP is expressed, G for only GFP is expressed, B for both RFP and GFP are expressed and N for none of the fluorescence marker is expressed (Melamed‐Bessudo *et al.* 2005)

Seeds resulting from BC1 have four different phenotypes, which can be observed by using GFP and RFP filters under florescent microscope, thus enabling to distinguish between the expressions of each maker separately (Figure 15 b). Since GFP/RFP can be homozygous and heterozygous, more phenotypes are supposed to be obtained from F2 seeds (see in Materials and methods). Homozygous (dark in green or dark

red or) are difficult to distinguish from heterozygous (light green or light red) by eyes precisely; thus we considered dark and light green/red as green/red only. In another words, we had four genotypes (green, red, both and none) instead of six genotypes (light green, light red, dark green, dark red, both and none). In the table below (1), the number of each phenotype from backcrosses (BC1) and self-pollination (F2) was described, respectively. In the BC1 generation, a total of 212 seeds were counted. The recombination rated obtained, based on the number of recombinant seeds (32 red and 27 green only seeds), which is 27.83%. In addition, the seeds resulting from self-pollination (F2) of 547 in total were also collected and analysed. The recombination rated in F2 is 23.58%.

Table 1: recombination rate of GFP and RFP as determined in the BC1 and F2

		Seed phenotype				
Generation	**Red**	**Green**	**Both**	**None**	**Total**	**% Rec.**
BC1	**3**	**27**	**69**	**84**	**212**	**27.83%**
(observed)	**2**					
F2	**69**	**60**	**315**	**103**	**547**	**23.58%**

From the data that we obtained, the recombination rate from BC1 which contains higher somatic homologous recombination rate also shows higher meiotic recombination rate, comparing to the wild type BC1, which is 25.3% (Hedatale 2010). However the difference is not significant (p=0.01). Since wild type F2 recombination rate was not tested, here we only included BC1 Chi-Square.

Table 2: Chi-square calculation of BC1

	Obseved	Expected
Recombinants	59	53.64
None-recombinants	153	158.4
Recombination rate	27.83%	25.30%
Chi-square	0.717	
Degree of freedom	1	
$P < 0.01$ (6.64) not significant		

The recombination frequency of F2 is 23.58%. We calculated that if the wild type meiotic recombination frequency is 20 %, the percentage of each genotype will be 16%, 9%, 9%, 66% in F2 population for None colour, green, red, both colour respectively (figure 17).

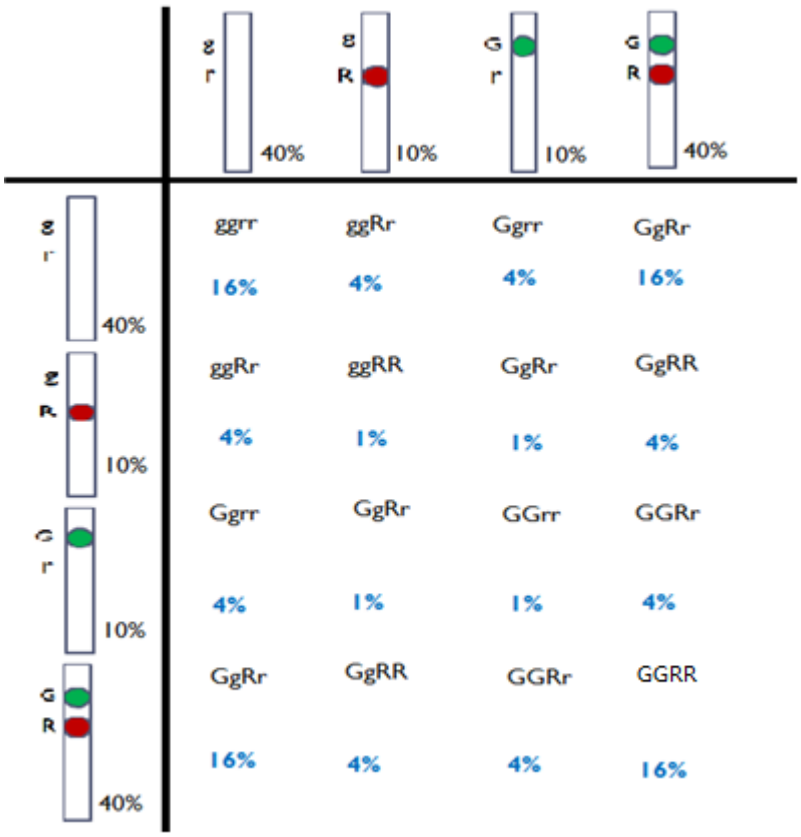


Figure 17: F2 population scheme, when the male meiotic recombination rate is 20%

Table 3 frequencies of each phenotype at different male meiotic recombination rate

R.F.	None	Green	Red	Both
10%	20.25%	2.25%	2.25%	75.25%
20%	16%	9%	9%	66%
30%	12.25%	12.75%	12.75%	62.25%
40%	9%	16%	16%	59%
50%	6.25%	18.75	18.75	56.25%

4. Discussion

2n gametes induction

Obtaining 2n gametes by skipping second meiotic division is essential for near-reverse breeding (Figure 2). However, the chance of influencing the desired moment is relatively low. A meiotic time-course for *Arabidopsis* pollen mother cells has been established and it has been shown that the overall duration of meiosis from the end of the meiotic S-phase to the tetrad stage was 33 hours (Armstrong *et al.* 2003). However, the remaining meiotic division stages are very rapid, finishing within 3 hours. Therefore, it is unpredictable which stages are interrupted by gas or heat treatment.

Therefore, different durations of gas treatment were performed in order to interrupt the second meiotic division within the 3 hours window. Even though 24 hours of treatment gave rise to 2n gametes, the result was difficult to reproduce. Compared to 24 hours of treatment, 39 and 48 hours of treatment showed more variations of plant development (no effects or plant death). This is because there are unexpected factors, such as plant stress, involved. High temperature combined with an exposure to high relatively humidity can affect plant development in *Arabidopsis* (Kim *et al.* 2001). In our case, we checked and recorded the humidity and temperature during the experiments which did not reach levels that were reported to cause abnormal formation of seeds. It has been reported that 1 day of continuous darkness resulted in abortion of siliques (SMITH and Stitt 2007). In order to find out whether darkness causes plant stress in our case, we have performed control experiments with continuous darkness for 24 and 48 hours. Interestingly, continuous darkness for 48 hours caused failure of seed formation while 24 hours of treatment had no effect. This might be because different *Arabidopsis* accessions and growing conditions were utilized compared to the study in the literature (SMITH and Stitt 2007). Plant stress effects like floral abortion in *Arabidopsis* can be caused by exposure to temperatures

above 40 ℃ (Warner and Erwin 2005). In our experiment, we observed that young Arabidopsis plants (3 weeks old) are less heat sensitive than older plants which have long bolts. It might be that the water transportation from root to shoot is more difficult if the bolts are too long under high temperature. Interestingly, exposure to 42 ℃ without watering for one day caused failure of flower formation. Moreover, Compression and decompression during gas treatment has a large effect of damaging of plant tissues. For example, while decreasing the pressure of the gas, the volume is increasing as the fact that volume * pressure is constant. Dramatic changes in pressure will lead to change of volume, thus damaging plant tissue by forming air bubbles in tissue, cells and vessels.

Although bigger pollen was obtained from gas and heat treatment, it is also difficult to confirm their identity as 2n pollen and even more difficult to identify if the bigger pollen were SDR gametes. In order to generate the bigger pollen we obtained into plants, we crossed them with the haploid inducer line CENH3. It is exploited that CENH3 can convert a natural tetraploid *Arabidopsis* into a diploid. The cross between big pollen and CENH3 allows us to generate the big pollen into plants or even SDR-0 plants once SDR gamete could be obtained. However, CENH3 haploid-inducer lines can only give rise to 34% haploid progeny when pollinated with wild-type plants (Ravi and Chan 2010). In our case, maximum 34% of progenies can possibly be SDR-0 plants when pollinated with treated F1 (crossed between Col and L*er*). Although CENH3 provides opportunities to obtain SDR-0 plants easily, the chance of obtaining SDR gametes by gas/heat treatment is very low. Next to this, low viability of 2n pollen compared to normal haploid pollen can be another difficulty in obtaining SDR-0 plants. It has been studied that pollen grain viability differs between genotypes and size of pollen grains in brassica, triploid F1 plants had a greatly reduced pollen viability by 24.52% (Schranz *et al.* 2006). Therefore, pollination took place between wild type pollen (n pollen) and the maternal donor plant in higher proportions.

We assumed that crosses between 2n pollen and male sterile Ler can give rise to triploid seeds and the size of seeds will be larger in comparison with wild type diploid seeds. In our experiments, we did not observe bigger or irregular seeds. The plants we obtained from the seeds did not show differences in flower sizes. If there were any triploid plants among offspring, we would observe bigger and sterile plants. Unfortunately the plants were not significantly differing in size, and only some of them were sterile. Normally sterility of plants can be an indication of triploid plants. In our case the male sterility is small, which might come from the F1 plants we used which were obtained by the cross between male sterile L*er* (MS/MS) and Col (ms/ms), thus F1 has change to be MS/ms of the male sterility. Therefore, the offspring from the cross between 2n pollen and male sterile L*er* can be male sterility as well. Since triploid plants were difficult to identify in our case, it was not feasible to prove that the 2n gametes were generated via the cross with male sterile Ler.

Although pollen stained with DAPI showed that the pollen have larger nuclei, and so higher DNA content indicating the possibility of 2n gametes, the genetic origin of the 2n gametes are still hard to distinguish. The stained pollen we observed was mostly at the same stage, the tri nucleus stage. The 2n pollen can be caused by first or second meiotic restitution.

Increasing meiotic recombination frequency (RF)

As mentioned in the introduction, high meiotic recombination can bring benefits to breeders, for example increasing the possibilities of breaking tightly linked genes, creating novel genotypes and restitution lines more easily. Somatic homologous recombination of plants shares the same pathways as meiotic homologous recombination (HR) in plans. This pathway can lead to crossover or none crossover events depending on the site of the double breaks. However, in our experiments observed a RF of 27.83% in the HR mutant and found 25.6% for the wild type

(Hedatale's results). Chi-square analysis showed that there is no significant difference in RF between the two.

The number of crossovers during meiosis is influenced by internal and external factors. Internal factors, such as genetic background, morphological and developmental differences, can cause differences in crossover incidences (Wijnker and de Jong 2008). Interestingly, there is a difference between the sexes in crossover frequencies both in plants and animals (Lenormand and Dutheil 2005). In *Arabidopsis* male meiosis shows more crossover incidents than female meiosis, which can be caused by different compaction states of chromatin during the meiosis phase (Wijnker and de Jong 2008). In our experiments, we observed higher crossover incidents in the BC1 (backcrossed with male sterile Ler) population but lower crossover incidents in F2. The male meiosis was taken into account in BC1 and both male and female meiosis were calculated in F2.

Meiotic recombination is initiated by double-stranded breaks in the DNA (DSBs) which performed by the spo11 protein. DSBs outnumber COs during meiosis in some organisms, which means a subset of DSBs are selected to form COs while the remainders is processed via the alternative pathway to form none COs (Globus and Keeney 2012). Meiosis is essential for sexual reproduction and CO defects can cause meiotic failure or gamete aneuploidy. Therefore cells have evolved systems ensuring that proper frequency and distributions of COs are accomplished (Globus and Keeney 2012). CO homeostasis is regulated to maintain appropriate number of COs per chromosome pair, this view has been supported in yeast, mice and C. elegans (Jones and Franklin 2006; Globus and Keeney 2012). It has been explored in spo11 mutants of budding yeast that crossover events were maintained at the same level as in wild type when DSBs were lowered by 20%, 30% and 80% (Martini *et al.* 2006). When increased DSBs in C. elegans (Yokoo *et al.* 2012), the COs were still maintained precisely six as wild type. Similarly, overexpressing SPO11 protein in mice showed

increased numbers of DSBs, but CO levels were maintained (Cole *et al.* 2012). Thus, both in yeast, worm and mice, cells have the ability to restrict the number of CO events during fluctuations in DSBs. Therefore we assume that in *Arabidopsis* the meiosis regulatory mechanism also plays a role in limiting the number of COs. This would explain that higher HR rates do not lead to higher CO rates.

5. Conclusions:

Near Reverse Breeding could be a useful approach to reproduce the heterozygous plant by two complementing homozygous lines which are SDR gametes. However, our attempts to induce SDR gametes by nitrous oxide under pressure treatment and heat treatment were so far not successful. Even though, bigger pollen was obtained from both treatments, the generation of bigger pollen remained difficult. The chemical treatments with colchicine and oryzalin did also not produce the expected bigger pollen in Arabidopsis plants.

Somatic homologous recombination is rare in plants and it shares the same path way as meiotic homologous recombination, resulting in crossover or none crossover. However, increased somatic homologous recombination could not increase crossover rate in our experiments.

References:

Akutsu, M., S. Kitamura, R. Toda, I. Miyajima and K. Okazaki (2007). "Production of 2n pollen of Asiatic hybrid lilies by nitrous oxide treatment." Euphytica **155**(1): 143-152.

Armstrong, S., F. Franklin and G. Jones (2003). "A meiotic time-course for Arabidopsis thaliana." Sexual Plant Reproduction **16**(3): 141-149.

Barba-Gonzalez, R., C. Miller, M. Ramanna and J. van Tuyl (2006). "Nitrous oxide induces 2n gametes in sterile F1 hybrids between Oriental × Asiatic lily (Lilium) hybrids and leads to intergenomic recombination." Euphytica **148**(3): 303-309.

Barrell, P. J. and U. Grossniklaus (2005). "Confocal microscopy of whole ovules for analysis of reproductive development: the elongate1 mutant affects meiosis II." The Plant Journal **43**(2): 309-320.

Bicknell, R. A. and A. M. Koltunow (2004). "Understanding Apomixis: Recent Advances and Remaining Conundrums." The Plant Cell Online **16**(suppl 1): S228-S245.

Bleuyard, J.-Y., M. E. Gallego and C. I. White (2006). "Recent advances in understanding of the DNA double-strand break repair machinery of plants." DNA repair **5**(1): 1-12.

Brayton, C. F. (1986). "Dimethyl sulfoxide (DMSO): a review." The Cornell Veterinarian **76**(1): 61.

Carputo, D., L. Frusciante and S. J. Peloquin (2003). "The Role of 2n Gametes and Endosperm Balance Number in the Origin and Evolution of Polyploids in the Tuber-Bearing Solanums." Genetics **163**(1): 287-294.

Cnudde, F. and T. Gerats (2005). "Meiosis: Inducing variation by reduction." Plant Biology **7**(4): 321-341.

Cole, F., L. Kauppi, J. Lange, I. Roig, R. Wang, S. Keeney and M. Jasin (2012). "Homeostatic control of recombination is implemented progressively in mouse meiosis." Nature Cell Biology.

d'Erfurth, I., S. Jolivet, N. Froger, O. Catrice, M. Novatchkova and R. Mercier (2009). "Turning Meiosis into Mitosis." PLoS Biol **7**(6): e1000124.

De Storme, N. and D. Geelen (2011). "The Arabidopsis mutant jason produces unreduced first division restitution male gametes through a parallel/fused spindle mechanism in meiosis II." Plant Physiology **155**(3): 1403-1415.

Dewitte, A., T. Eeckhaut, J. Van Huylenbroeck and E. Van Bockstaele (2010). "Induction of 2n pollen formation in Begonia by trifluralin and N2O treatments." Euphytica **171**(2): 283-293.

Dirks, R., K. Van Dun, C. B. De Snoo, M. Van Den Berg, C. L. C. Lelivelt, W. Voermans, L. Woudenberg, J. P. C. De Wit, K. Reinink, J. W. Schut, E. Van Der Zeeuw, A. Vogelaar, G. Freymark, E. W. Gutteling, M. N. Keppel, P. Van Drongelen, M.

Kieny, P. Ellul, A. Touraev, H. Ma, H. De Jong and E. Wijnker (2009). "Reverse breeding: a novel breeding approach based on engineered meiosis." Plant Biotechnology Journal **7**(9): 837-845.

Dirks, R. H. G., C. M. P. Van Dun and K. Reinink (2003). Reverse Breeding. R. Z. Z. e. Z. B.V.

Drouaud, J., C. Camilleri, P. Y. Bourguignon, A. Canaguier, A. Bérard, D. Vezon, S. Giancola, D. Brunel, V. Colot and B. Prum (2006). "Variation in crossing-over rates across chromosome 4 of Arabidopsis thaliana reveals the presence of meiotic recombination “hot spots”." Genome research **16**(1): 106-114.

Gerstein, M. B., C. Bruce, J. S. Rozowsky, D. Zheng, J. Du, J. O. Korbel, O. Emanuelsson, Z. D. Zhang, S. Weissman and M. Snyder (2007). "What is a gene, post-ENCODE? History and updated definition." Genome research **17**(6): 669-681.

Gherbi, H., M. E. Gallego, N. Jalut, J. M. Lucht, B. Hohn and C. I. White (2001). "Homologous recombination in planta is stimulated in the absence of Rad50." EMBO reports **2**(4): 287-291.

Globus, Samuel T. and S. Keeney (2012). "The Joy of Six: How to Control Your Crossovers." Cell **149**(1): 11-12.

Hedatale, V. (2010). Genes involved in meiotic recombination in *Petunia* hybrid and *Arabidopsis thaliana*. PhD, Radboud University.

Jones, G. H. and F. C. H. Franklin (2006). "Meiotic crossing-over: obligation and interference." Cell **126**(2): 246-248.

Kim, S. Y., C. B. Hong and I. Lee (2001). "Heat Shock Stress Causes Stage-specific Male Sterility in <i>Arabidopsis thaliana</i&gt." Journal of Plant Research **114**(3): 301-307.

Kim, S. Y., C. B. Hong and I. Lee (2001). "Heat shock stress causes stage-specific male sterility in Arabidopsis thaliana." Journal of Plant Research **114**(3): 301-307.

Kitamura, S., M. Akutsu and K. Okazaki (2009). "Mechanism of action of nitrous oxide gas applied as a polyploidizing agent during meiosis in lilies." Sexual Plant Reproduction **22**(1): 9-14.

Lenormand, T. and J. Dutheil (2005). "Recombination difference between sexes: a role for haploid selection." PLoS biology **3**(3): e63.

Lieberman-Lazarovich, M. and A. A. Levy (2011). "Homologous Recombination in Plants: An Antireview." Plant Chromosome Engineering Methods and Protocols Methods in Molecular Biology **701**: 51-65.

Lindhout, B. I., J. E. Pinas, P. J. J. Hooykaas and B. J. Van Der Zaal (2006). "Employing libraries of zinc finger artificial transcription factors to screen for homologous recombination mutants in Arabidopsis." The Plant Journal **48**(3): 475-483.

Marimuthu, M. P. A., S. Jolivet, M. Ravi, L. Pereira, J. N. Davda, L. Cromer, L. Wang, F. Nogué, S. W. L. Chan, I. Siddiqi and R. Mercier (2011). "Synthetic Clonal Reproduction Through Seeds." Science **331**(6019): 876.

Martini, E., R. L. Diaz, N. Hunter and S. Keeney (2006). "Crossover homeostasis in yeast meiosis." Cell **126**(2): 285-295.

Matsushima, R., L. Y. Tang, L. Zhang, H. Yamada, D. Twell and W. Sakamoto (2011). "A conserved, Mg2+-dependent exonuclease degrades organelle DNA during Arabidopsis pollen development." The Plant Cell Online **23**(4): 1608-1624.

Melamed‐Bessudo, C., E. Yehuda, A. R. Stuitje and A. A. Levy (2005). "A new seed‐based assay for meiotic recombination in Arabidopsis thaliana." The Plant Journal **43**(3): 458-466.

Mezard, C. (2006). "Meiotic recombination hotspots in plants." Biochemical Society Transactions **34**(4): 531-534.

Nakamura, M., Naoi, K., Shoji, T., and Hashimoto, T. (2004). Low concentrations of propyzamide and oryzalin alter microtubule dynamics in Arabidopsis epidermal cells. Plant and cell physiology 45, 1330-1334.

Negri, V. and G. Lemmi (1998). "Effect of selection and temperature stress on the production of 2n gametes in Lotus tenuis." Plant Breeding **117**(4): 345-349.

Okazaki, K., K. Kurimoto, I. Miyajima, A. Enami, H. Mizuochi, Y. Matsumoto and H. Ohya (2005). "Induction of 2n pollen in tulips by arresting the meiotic process with nitrous oxide gas " Euphytica **143**(1): 101-114.

Paredez, A.R., Persson, S., Ehrhardt, D.W., and Somerville, C.R. (2008). Genetic evidence that cellulose synthase activity influences microtubule cortical array organization. Plant physiology 147, 1723-1734.

Pécrix, Y., G. Rallo, H. Folzer, M. Cigna, S. Gudin and M. Le Bris (2011). "Polyploidization mechanisms: temperature environment can induce diploid gamete formation in Rosa sp." Journal of Experimental Botany **62**(10): 3587-3597.

Perotti, E., D. Grimanelli, P. John, D. Hoisington and O. Leblanc (2004). Why is transferring apomixis to crops still a dream. 4th International Crop Science T. Fischer, N. Turner, L. McIntyreet al. Australia, The Regional Institute Ltd.

Ravi, M. and S. W. L. Chan (2010). "Haploid plants produced by centromere-mediated genome elimination." Nature **464**(7288): 615-618.

Schranz, M. E., L. Kantama, H. De Jong and T. Mitchell-Olds (2006). "Asexual reproduction in a close relative of Arabidopsis: a genetic investigation of apomixis in Boechera (Brassicaceae)." New Phytologist **171**(2): 425-438.

Shinohara, M., S. D. Oh, N. Hunter and A. Shinohara (2008). "Crossover assurance and crossover interference are distinctly regulated by the ZMM proteins during yeast meiosis." Nature genetics **40**(3): 299-309.

SMITH, A. M. and M. Stitt (2007). "Coordination of carbon supply and plant growth." Plant, Cell & Environment **30**(9): 1126-1149.

Swoboda, P., S. Gal, B. Hohn and H. Puchta (1994). "Intrachromosomal homologous recombination in whole plants." The EMBO journal **13**(2): 484.

Van Dun, C. M. P. and R. H. G. Dirks (2008). Near Reverse Breeding. United States, 20080098496.

Veilleux, R. E. and F. I. Lauer (1981). "Variation for 2n pollen production in clones of <i>Solanum phureja Juz. and Buk." TAG Theoretical and Applied Genetics **59**(2): 95-100.

Warner, R. M. and J. E. Erwin (2005). "Naturally occurring variation in high temperature induced floral bud abortion across Arabidopsis thaliana accessions." Plant, Cell & Environment **28**(10): 1255-1266.

Weiss, H. and J. Maluszynska (2001). "Chromosomal rearrangement in autotetraploid plants of Arabidopsis thaliana." Hereditas **133**(3): 255-261.

Wijnker, E. and H. de Jong (2008). "Managing meiotic recombination in plant breeding." Trends in Plant Science **13**(12): 640-646.

Wijnker, E., K. van Dun, C. B. de Snoo, C. L. Lelivelt, J. J. Keurentjes, N. S. Naharudin, M. Ravi, S. W. Chan, H. de Jong and R. Dirks (2012). "Reverse breeding in Arabidopsis thaliana generates homozygous parental lines from a heterozygous plant." Nature genetics **44**(4): 467-470.

Woodward, D. (2009). "Heredity and Life Cycles ", from https://wikispaces.psu.edu/display/110Master/Heredity+and+Life+Cycles.

Yokoo, R., K. A. Zawadzki, K. Nabeshima, M. Drake, S. Arur and A. M. Villeneuve (2012). "COSA-1 Reveals Robust Homeostasis and Separable Licensing and Reinforcement Steps Governing Meiotic Crossovers." Cell **149**(1): 75-87.