Pilot study of Near-Reverse Breeding in *Arabidopsis thaliana*

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Abbreviations:
2.4D: 2.4-dichlorophenoxyacetic acid
2n: Diploid genotype
CENH3: Centromeric histone mutant
cM: Centimorgan
Col: Columbia
Cvi: Cape Verde Islands
DSB: Double-strand break
DH: Doubled-haploid
EMS: Ethyl methane sulfonate
FDR: First Division Restitution
GEM: Genome Elimination caused by Mix of CENH3 variants
GFP: Green fluorescence protein
GMO: Genetically modified organism
KASP: Kbioscience Competitive Allele-Specific PCR genotyping system
kb: Kilo base
L*er*: Landsberg *erecta*
Mb: Mega base
MES: 2-(N-morpholino) ethanesulfonic acid
MS: Murashige & Skoog
MQ: Milli-Q
n: Haploid genotype
N_2O: Nitrous Oxide
NRB: Near-Reverse Breeding
ρ: Pressure
PCR: Polymerase chain reaction
qrt-1: *Quartet*-1
QTL: Quantitative trait locus
RB: Reverse Breeding
RNAi ribonucleic acid interference
SDR: Second Division Restitution
siRNA: small interfering ribonucleic acid
SNP: Single-nucleotide polymorphism
V: Volume
x: Number of chromosomes

Abstract

Plant breeding exists since man began to domesticate its plants for own consumption and that of its husbandry. Hybrid breeding is from great importance in plant breeding due to vigorous heterozygous (hybrids) which are superior growth and yield compared with their homozygous parents. But hybrid genotypes cannot be maintained or fixed due to allele segregation. Techniques are available to reconstruct heterozygous plants, but are mostly expensive, will take a lot of generations or genetic modification is necessary.

Near-Reverse Breeding is a new technique for the reconstruction of heterozygous plants, which makes use of unreduced gametes resulting from equational or second division restitution (SDR). SDR plants are generated and selected to be complementing with the use of SNP markers. DHs are made from the complementing SDRs. The most complementing DHs are selected to reconstruct the starting heterozygote. In this pilot study, the success rate of Near-Reverse Breeding is researched in the model crop *Arabidopsis thaliana*, whereby this technique should prove that heterozygous plants could be maintained and obtained in a short period. The F1 hybrid Col x L*er* in the *quartet*-1 background is crossed with the homozygous Cvi-0 to obtain four progenies of one single meiosis. Using these four progenies, the meiosis and also the SDR gametes could be reconstructed. For Near-Reverse Breeding, SDR gametes should be produced. In lily and tulip these gametes are produced with N_2O under high pressure. But is this technique also useful in *Arabidopsis*?

The 36 sets of four offspring resulting from one meiosis give SDR gametes which showed 53% homozygosity. If complementing SDR gametes are selected on average 38% of heterozygosity is already fixed in Near-Reverse Breeding. Before complementing SDR-0s were selected for reconstruction of the starting hybrid, SDR gametes and SDR-0 plants have to be induced. With nitrous oxide under high pressure, larger pollen could be produced, which are 2n gametes. These 2n gametes are transferred to a haploid inducer to produce SDR plants. But no SDR-0 plants were observed.

Near-Reverse Breeding could be a useful approach to reproduce the heterozygous plant with two complementing homozygous lines with the use of SDR gametes, theoretically. But no SDR plants are produced, which are essential for Near-Reverse Breeding. So the focus of further research should lay on a high efficiency of SDR gamete production and a more efficient method to induce these SDR gametes to SDR-0 plants.

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Introduction

Plant breeding exists since man began to domesticate its plants for own consumption and that of its husbandry. The state-of-the-art breeding, as we know it nowadays, using sophisticated genetics, molecular and statistical methods started only few decades ago. Under optimised conditions, breeders select the best performing plants, make crosses and select again new parents among their offspring. After consecutive generations of crossing and selections families are produced that are true-bred for specific traits, but often displayed poor growth and small size of the plant and its organs. People then observed that crosses between different inbreds produce vigorous heterozygous (hybrids) with superior growth and yield. Apparently, the combination of different genotypes of the two parents in a hybrid excels their homozygous parents in all respects.

Hybrid genotypes cannot be maintained or fixed due to allele segregation in the offspring. Breeders therefore produce inbred lines as parents for producing large amounts of F1 seeds and select the best performing parent combination for commercial use. This method is known as *forward* or *traditional breeding* (Dirks 2003) and is very laborious and time consuming as it takes 8-12 years of crossings and selections appropriate parents. The most frequently used technique is described as recurrent selfing. Two complementing parental lines are produced by 8-12 generations of self-pollination of the hybrid. Crossing these complementing lines reproduces the heterozygous F1 plant intended as commercial cultivar. The formula $(1/2)^{(n-1)}$, where n is the number of population, gives an indication about the degree of heterozygosity of each generation. The proportion of heterozygosity in the F6 population is $(1/2)^{(6-1)}$ is 3% (97% homozygous) (Allendorf 2006). As said, recurrent selfing takes many generations to obtain appropriate parents to produce hybrids.

But there are alternatives for maintaining heterozygous plants, like vegetative propagation of bulbs, roots, tubers, stems etc. These methods are used mostly for polyploidy, biannual or perennial crops like potato, banana and sugarcane, where traditional breeding is virtually impossible. A second method is apomixis, a natural method of clonal seed production (Nogler 1984), so without the contribution of the male gamete, giving progeny that are (almost always) exact genetic copies of the mother parent. Apomixis occurs in more than 400 species, of which most belong to the Asteraceae, Poaceae, and Rosaceae families and almost always in polyploid forms. The trait is, apart from few exceptions, absent in to crop species or their wild relatives. Isolating the genes for apomixis and transferring them in crops by genetic engineering sounds promising, but is as yet not a wide-spread technology due to the complexity of the trait, the polyploid nature of most natural apomicts and the indispensible need of GMO technology. Ravi et al. (Marimuthu 2011) was the first to claim synthetic apomixis by introducing apomeiosis (first division restitution) in a mutated *DYAD*/*SWITCH*1 *Arabidopsis* that produce unreduced gametes at full maternal.

An entirely novel strategy, *Reverse Breeding*, turns the process of traditional breeding upside down in a way that superior hybrids rather than their parents are now the starting material for producing large amounts of F1 seeds in a fast and direct way. Selecting hybrids instead of selecting homozygous plants is an entirely new strategy in plant breeding and has the advantage of more flexibility in combining desirable parental traits in heterozygous plants while speeding up plant breeding enormously (Dirks 2003). For this technique meiotic recombination complexity is strongly reduced by knocking

down crossovers at meiotic prophase I. The remaining meiotic recombination is then confined to whole chromosomes that assort randomly at metaphase I – anaphase I, and so is proportional to the number of chromosome pairs in the cell complement. Crossovers can be suppressed by different post-transcriptional inhibition pathways (Wikipedia 2010), like RNAi, dominant-negative mutation and siRNA technologies, and will lead to non-recombinant chromosomes in their gametes. The spores thus formed can be regenerated and their genome doubled forming so-called doubled haploids (DHs).

Reverse Breeding was first developed and tested in the small flowering model plant *Arabidopsis thaliana*, which has a small genome size of 157 Mb (Bennett 2003), a fully saturated linkage map, low chromosome number of x=5 and a short life cycle of 6-8 weeks. Transformation of RNAi constructs was possible, but regeneration of the spores had to wait for the haploid regeneration technology based on the frequently published CENH3 modified haploid inducer (Ravi 2010). The CENH3 plant produce normal meiosis, but the maternal chromosomes upon fertilization degenerate forming a paternal haploid, that later doubles to a dihaploid plant. The procedure of this technique is shortly summarised in figure 1. Reverse Breeding can now make use of these technologies for the reconstruction of complementary homozygous parents derived from a heterozygous individual. The use of GMO is a drawback of limited significance as absence of transgenes in the parental chromosomes can be easily selected for. A second, more important disadvantage is the exponential decrease of non-recombinant complementary parents in species with higher chromosome numbers, following $½^n$, in which n=haploid chromosome number (Dirks 2003; Dun 2009).

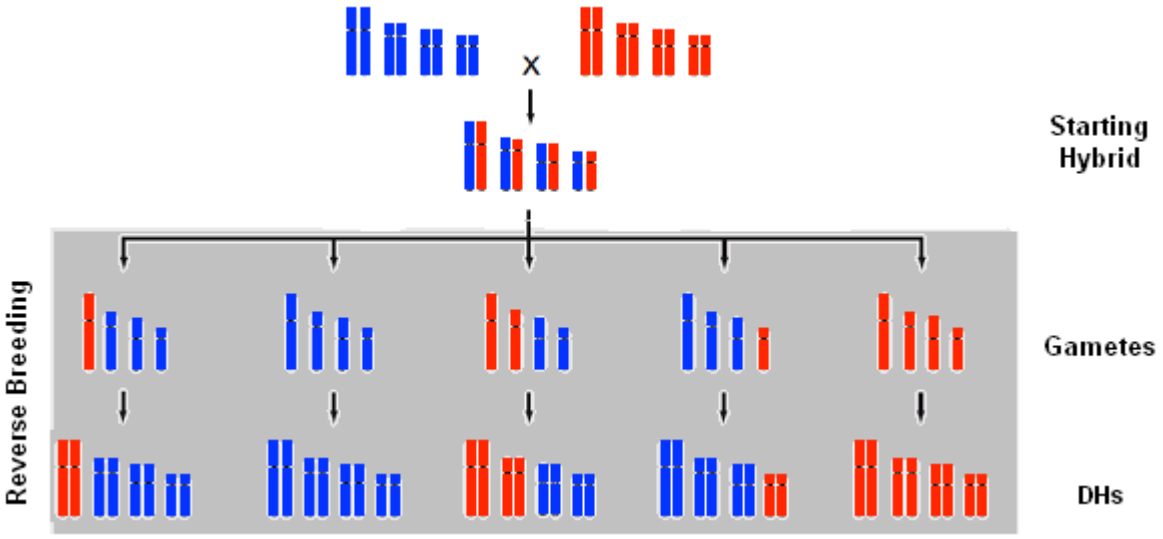

Figure 1 The Reverse Breeding approach, whereby the starting hybrid produces non recombinant chromosomes. Doubled haploids were produced from these gametes, where after complementing DHs are selected to reconstruct the starting hybrid (Dirks 2003).

A second method to reduce heterozygosity in the offspring of a heterozygous F1 is *Near-Reverse Breeding* (NRB), which makes use of unreduced gametes resulting from equational or second division restitution (SDR). It means that these cells have skipped anaphase II and so keep homozygosity for the chromosome regions between centromere and a proximal crossover event if one crossover per chromosome arm occurs (Dirks 2006; Dun 2008). In a simplified scenario with none or one crossover per chromosome arm, only the distal parts of one arm or either arms are heterozygous. In an arm with

two or three crossover, this situation is more complicated. In general it is estimated that SDR lead to 60% loss of heterozygosity (Carputo 2006). Figure 2 shows schematically the concept of Near-Reverse Breeding. By the use of marker assisted selection, the most complementing lines are selected. Doubled haploids are produced from these lines and are tested by marker assisted selection for the most complementing lines. The level of homozygosity can eventually be increased by self-pollination. Near-Reverse Breeding will always lead to residual heterozygosity – that is why it is "near" – but two consecutive sexual generations with SDR regeneration will lead to very low levels of heterozygosity. NRB is also advantageous in using natural methods rather than GMO technology and it suffers less from chromosome number effects compared with Reverse Breeding; speeds up backcrossing and can produce small introgressions, called heterogeneous inbred families, for QTL and plant trait studies (Dun 2008; Dirks 2009; Weerenbeck 2009). There are four technical bottlenecks to consider: 1) induction or enhancement of natural SDR meiosis by genetic or environmental means; 2) isolation or enrichment of pollen fractions with unreduced spores by separating 2n- and n-pollen; 3) regeneration in vitro or with the above mentioned CENH3 haploid inducer of 2n-pollen to diploid SDR-0 plants; 4) genotyping the SDR plants with a set of co-dominant PCR-markers. In this pilot study we will work out and test several aspects of the NRB concept in the model species *Arabidopsis*.

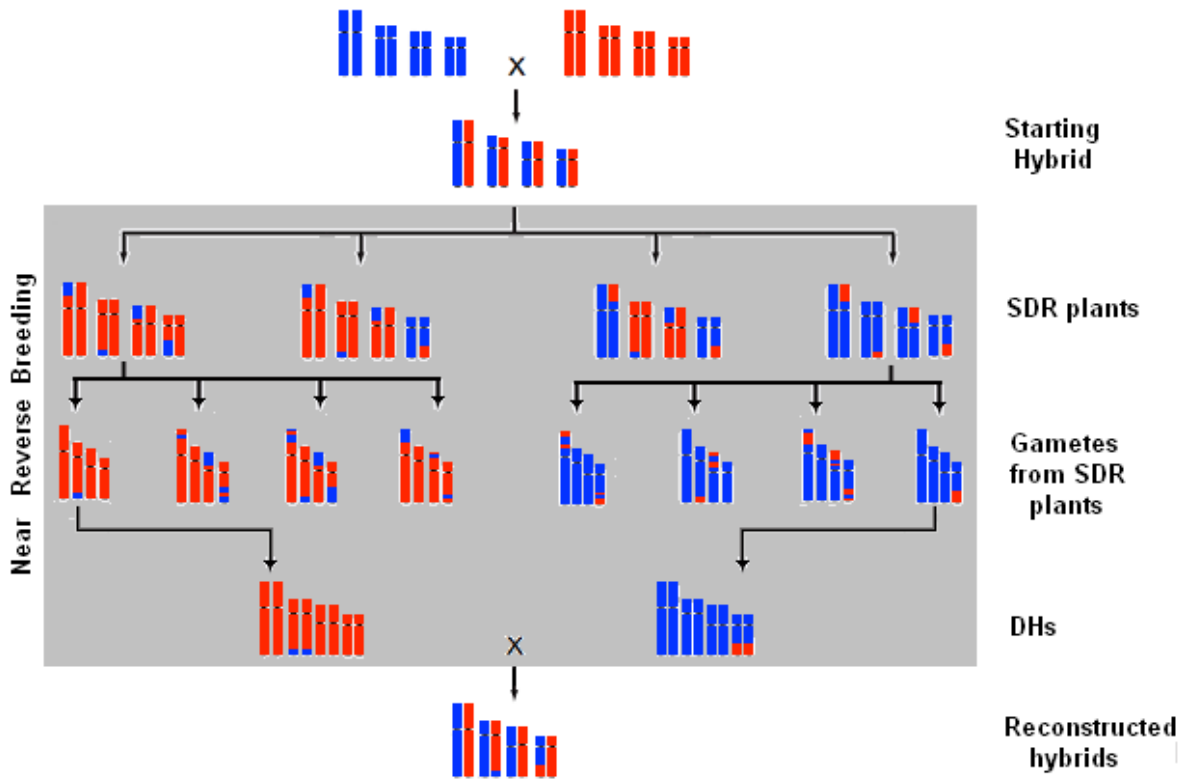

Figure 2 The Near-Reverse Breeding approach, whereby SDR gametes / plants are produced of the starting hybrid. Doubled haploids are produced after a haploid induction of the SDRs, so heterozygosity is lost. The goal is to approve to almost complementing homozygous lines, so the starting hybrid is reconstructed (Dun 2006).

Arabidopsis thaliana is a model plant to test the power and possibilities of Near-Reverse Breeding. This crop has a short life cycle (6-8 weeks), a small genome, which is sequenced totally. In addition to its superior genetics and genomics mentioned above, *Arabidopsis* has a collection of unique mutants, most powerful for this kind of work. Firstly, the *quartet*-1 mutant is important as it keeps the four spores of a single male

meiosis together in the tetrad allowing single meiosis analysis of unordered tetrads, a feature found only in fungi and mosses (Preuss 1994; Francis 2006). This mutant could be interesting for determining the positions of crossover events in a single meiosis. Secondly, *Arabidopsis* has male sterility mutants, which is imperative for certain crossings as the species is by nature a self-pollinator. We chose the male sterility mutant derived from an EMS population. This mutant will produce little or no pollen and is incapable of fertilization (Peirson 1996). A plant with the *quartet*-1 background crossed with a male sterile plant could generate the four gametes of a single meiosis, whereby information about the meiosis could be generated, like recombination patterns and amount of recombination.

The third *Arabidopsis* mutant of interest is the centromeric histone mutant (CENH3), which will degenerate its own female genome at zygote stage, but upon pollination will let the male sperm develop in its own cytoplasm and so makes it possible to induce haploid embryo's that later develop into (di)haploid plants (Ravi 2010). A related very recent technology is GEM (Genome Elimination by Mix of CENH3 variants). The GEM mutant should be more efficient than CENH3, because of the gene pyramiding of the mix of CENH3 variants (Marimuthu 2011). According to Ravi (2010), genome elimination does not always take place and the CENH3 and GEM constructs produce diploids, aneuploids and haploids (25-45% with CENH3) (Marimuthu 2011). With the limitations mentioned we can use this haploid inducer for the generation of haploids and SDR-0 plants in our experiments.

As mentioned before, SDR meiosis is essential for the production of 2n gametes with high levels of homozygosity. This shortcut in the process of meiotic division can be induced by mutation, heat or cold shocks and chemicals (Brownfield 2010). In lily and tulip, nitrous oxide (N_2O) is used as gas under high pressure to act as a disturber of the spindle formation during meiosis, whereby 2n gametes are produced for inducing higher ploidy level in the progeny (Barba-Gonzalez 2006; Akutsu 2007; Kitamura 2009). The nitrous oxide treatment is used for the production of SDR gametes in *Arabidopsis*.

Genotyping is an important tool to identify complementing lines and establish SDR gametes and map crossover events. The genotyping is done by the use of Single Nucleotide Polymorphisms (SNPs), which are small abbreviations between different genotypes. In *Arabidopsis* a lot SNPs are available. With the use of the *Arabidopsis* plant in the *quartet*-1 background and the availability of SNPs, information about the meiosis could be obtained, like recombination and crossovers. Meiosis is an important process in Near-Reverse Breeding for the production of recombination and SDR gametes.

Near-Reverse Breeding is a new technique for the reconstruction of heterozygous plants. The theoretical operations are written down, but the practical implementation should be still proven. In this pilot study, the success rate of Near-Reverse Breeding is researched in the model crop *Arabidopsis thaliana*, whereby this technique should prove that heterozygous plants could be maintained and obtained in a short period. The production of SDR gametes and regeneration to SDR-0 plants are essential for Near-Reverse Breeding. Nitrous oxide under high pressure is used as tool to produce the SDR gametes (2n) and these gametes will be generated to diploid plants with the CENH3. Besides the production of SDR gametes and second study is started. An F1 hybrid in the *quartet*-1 background is used to produce four progeny derived from a single meiosis. Therefore the recombination patterns due to crossovers could be observed with SNP analysis. With these four haploid sets, the meiosis and SDR gametes could be

reconstructed. Using these data, calculations could be made about the amount of homozygosity in the SDR plants. Besides the amount of fixed heterozygosity in reconstructed complementing SDR-0 plants could be calculated. With the production of SDR gametes with nitrous oxide and the reconstruction of SDR gametes, the success rate of Near-Reverse Breeding could be proven.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana accessions Columbia (Col), Landsberg *erecta* (L*er*), a male sterile (mutated gene is unknown) Cape Verdi Island (Cvi-0) derived from an EMS population, the *Quartet*-1 (*qrt*-1/*qrt*-1) in both Col and L*er* background and two haploid inducers (GEM and CENH3) were used. Where the plant material was obtained from could be found in the **supplementary data**. After a maturation period of minimum two weeks after seed harvest, seeds were incubated on a piece of moistened filter paper containing 600 µL 10mM KNO_3 in a 51 x 15 mm petridish sealed with parafilm. Seeds were treated at 4°C to break dormancy and ensure synchronising germination for the duration of 3-7 days. The petridish with the seeds were transferred to a climate chamber (photoperiod 16 hours of light: 8 hours of dark at 20 °C) for germination for two days. Germinated seeds were transferred by a wet brush to a 51-wells tray (30 x 50 cm) containing soil and this was kept in the climate chamber (photoperiod 16 hours of light: 8 hours of dark at 20°C), where the plants were reared till flowering.

Crossings

The following crossings were made:

1. F1 hybrid from a cross between Col and L*er*, both of them recessive for *quartet*-1 mutant (*qrt*-1 *qrt*-1) and both of them as wild type Col and L*er*.

2. Male sterile Cvi-0 (f) x F1 hybrid (Col x L*er*) in *quartet*-1 background (m)

3. CENH3 (f) x F1 hybrid wild type (Col x L*er*) treated with nitrous oxide gas for 2n pollen (m)

4. CENH3 x F1 hybrid (Col x L*er*) and GEM x F1 hybrid (Col x L*er*), where the F1 hybrid is in the *quartet*-1 mutant and the wild type F1 hybrid.

We emasculated the flowers one or two days before crosses were made. First mature siliques and open flowers were removed. Then the six stamens of a still unopened flower bud were eliminated under the dissecting microscope with a cleaned (by 70% ethanol) pair of tweezers. The flowers of the male sterile Cvi-0 did not need to be emasculated due to the absence of (viable) pollen. We made crosses by tapping the stamens of the male flowers with a pair of tweezers on a slide. Under the light microscope, individual pollen or pollen tetrads were picked up separately with a horse hair cleaved on a paperclip and placed onto the pistil of a virgin flower. Flowers, which were used as female, were marked. Seeds could be harvest from the plants after 2.5 weeks of crossings and stored for at least 2 weeks.

Haploids were produced by crosses with the CENH3 or GEM. Crosses were made with CENH3 and GEM both as female as male. Besides CENH3 and GEM was also used as female for crosses with the F1 hybrid treated with nitrous oxide, to produce SDR-0 plants. Genome elimination of the haploid inducer took place upon zygote formation. The presence of aneuploidy could cause a high level of seed abortion. KASPAR probes (explained later in M&M) was used to check the progeny of the crosses were haploids or diploids.

Sowing
The seeds of the second cross (with Cvi-0 and F1 hybrid in the *quartet*-1 background) were treated as described above with the following modification: after germination, the seeds were transferred to an agar plate containing an autoclaved ½ MS including vitamins (2.30 g/L) and 8.0 g/L Daishin agar solution (pH = 5.8). The exact descriptions of all the substances can be found in the **supplementary data**. The agar plates with germinated seeds were put in the same climate chamber, where they coming from. Plants were transferred with a wet brush to rockwool blocks (40 x 40 mm) in climate cell (photoperiod 11 hours of light / 13 hours of dark at 20 °C) after producing their first leaves and some rootlets

The seeds of the third and fourth crossing were obtained from pollinations with the genome eliminators. Seeds were kept for a longer period in tissue culture to reach higher germination rates of these crosses. Therefore the seeds needed to be sterilized to kill all germs. Seeds coming from a cross with the genome eliminators were put in 1.5 mL Eppendorf tubes. The tubes were placed with open clip into a rack inside a dessicator jar placed in the fume hood. The jar contained two 100 mL beakers with both 50 mL bleach (NaOCl) and 1.5 mL concentrated HCl. Chlorine gas was produced, whereby the seeds were sterilized. After the sterilization process of three hours, the tubes were transferred to the flow chamber with closed clips. The tube was reopened to release the chlorine gas in the flow chamber. After one hour, the seeds were divided in 94 x 15 mm petri dishes with autoclaved ½ MS mediums including vitamins (2.3 g/L) containing 10 g/L sucrose, 0.50 g/L MES and 8.0 g/L Daishin agar (pH = 5.8)). The petri dishes with seeds were sealed with tape and grown at 4 °C for 7 days. Where after the seeds were transferred to a climate chamber (photoperiod 16 hours of light: 8 hours of dark at 20°C) for germination for two weeks, before they were planted on soil in a climate cell (photoperiod 16 hours of light: 8 hours of dark at 20°C).

Tissue culture
We used embryo rescue to reach higher emergence and produce more plant material of a single plant than by seed sowing. Siliques were cut from the plant after 12 days of crossing and put in a 1.5 mL eppendorf tube. Siliques were sterilized by washing them with 1.0 mL 70% ethanol (C_2H_6O) for ten seconds. After that, the ethanol was replaced by 1.0 mL bleach (NaOCl) with 10% tween-20 for ten minutes. The proportion for both liquids was respectively 970 µL bleach and 30 µL 10% tween-20. Siliques were rinsed by inserting 1.0 mL autoclaved MQ water three times. The sterilization process was accomplished by drying the siliques on sterile filter paper. Siliques were transferred one by one on agar, where they were cut open along the replum with syringe needles. We carefully scraped out the ovules with a needle and the membrane of the embryo was removed. The embryo was transferred to a 24 wells plate, where each well contain 1.0 mL of ½ MS solution (2.30 g/L MS incl. vitamins, 10 g/L sucrose, 0.50 g/L MES (pH = 5.8)) and added 1.0 mM 2.4 D (2.4-dichlorophenoxyacetic acid)). The 24 wells plate was sealed and placed in the climate chamber (photoperiod 16 hours of light: 8 hours of dark at 20°C) on a G10 Gyrotory Shaker. Every two weeks the medium was refreshed. When the embryos reached the preferable size, the embryos were transferred to 94 x 15 mm petridishes containing ½ MS medium (2.30 g/L MS incl. vitamins, 10 g/L sucrose, 0.50 g/L MES and 8.0 g/L Daishin agar (pH = 5.8)), which was also refreshed every two weeks. In the petri dish the embryos fulfilled their growth to plants, where after they were transferred to soil.

Producing of SDR gametes

2n pollen had to be induced by a nitrous oxide treatment under high pressure, before SDR (2n) pollen could be crossed with CENH3 or GEM plants. F1 hybrids of Col x L*er*, which made the transition from vegetative to inflorescence meristem with the first flowers open, were used for 2n pollen production. The plants were put in a pressure cylinder, where the pressure of N_2O increased from 0 to 6 bars in 1 to 1.5 hours. The treatment took 24 hours and was executed by a temperature of 13 °C. After 24 hours, the pressure was decreased slowly (duration was 8 hours) and the plants were transferred back to the climate cell. The size of the pollen of the treated plants was observed under the dissecting microscope after 5 to 7 days. The stamens were tapped on slides with a pair of tweezers. Lactophenol acid fuchsin was eventually used to stain the pollen for measurements, but hereby the pollen were useless for crosses by pollen dead. Pollen was also observed without lactophenol acid fuchsin, so if larger pollen were observed, crosses could be made with a genome eliminator.

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, plant tissue (leaves or callus) were taken from the *Arabidopsis* plants (5 to 15 mg / plant) and placed in a 96 wells plate containing 100 µL of lysis buffer in each well. The Breeding company Rijk Zwaan did the genotyping. They screened the plant tissue for in total 36 SNP markers divided over the 5 chromosomes (depicted below).

Chromosome 1:	10 markers	/	30,427,671 bp
Chromosome 2:	6 markers	/	19,698,289 bp
Chromosome 3:	7 markers	/	23,456,830 bp
Chromosome 4:	6 markers	/	18,585,056 bp
Chromosome 5:	7 markers	/	26,975,502 bp
Total:	36 markers	/	119,143,348 bp

Rijk Zwaan used the KASP genotyping system as technique to observe the SNPs. The SNP analysis is based on PCR. In the first phase of the PCR, two primers are used per SNP. One will bind on the appropriate allele and the other will bind on the complementary region. Fluor labelled oligo is bound to a quencher, so they could not give a fluorescent signal. The fluor labelled oligo will correspond with one of the amplified alleles, whereby the oligo is no longer quenched and will give a fluorescence signal. For both primers an oligo is present. If the SNP is homozygous, only one fluorescence signal is generated. If it is heterozygous a mixed fluorescence signal is observed (Robinson 2011).

The starting material (Col, L*er* and male sterile Cvi-0) was genotyped like the above described method. The results of the SNP analysis are shown in figure 3. With the results of the genotyping of the progeny of the cross between the male sterile Cvi-0 and the F1 hybrid (Col x L*er*) in the *quartet*-1 background could be reconstructed. The results were lined next to the genotype of the male sterile Cvi-0. The progeny of the cross between the Cvi-0 and F1 hybrid contains two strands. One of these strands is derived from the Cvi-0, which scores always the same due to the homozygosity of this plant and so recombination could not be observed. If the progeny of the cross and the Cvi-0 are lined up next to each other the genotype of the F1 hybrid could be reconstructed with the use of figure 4, the possible combinations are given with also the scoring of the F1 hybrid.

Besides, genotyping could be used for calculating the recombination and the amount of homo- and heterozygosity of SDRs, FDRs and the F2 population in percentages. The distance between the markers was known in base pairs. We used the middle between the markers as reference point where the crossover occurred. So the number of base pairs, which were recombinant and non-recombinant, could be calculated (see also the **supplementary data** for the calculations).

Figure 3 The results of SNP analysis of the starting material. In total 36 SNP markers were used. The black markers are (near to) the centromeres. The pink markers are markers on the short arm. The markers on the long arm are light blue. Two markers for male sterile Cvi-0 were undefined.

	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
Cvi-0	B	B	B	A	A	A	B	A	A	B	B	B	B	B	A	B	B	B	B	A	B	B	B	B	B	A	A	B	B	B	B	A	-	-	B	A

Progeny of the crossing between the male sterile Cvi-0 and the F1 hybrid (Col x L*er*) in the *quartet*-1 mutant should be sequenced to observe gene conversions. For each genotype, at least 1 g fresh plant tissue was necessary for sequencing. Therefore the plants were put in a climate cell with short day circumstances to stimulate the vegetative growth and delay flowering, whereby more plant tissue was produced. If the plant had produced his first flowers, the plant was harvested. The plant was pulled of the rockwool and roots and the stem were cut. The rosette leaves were assembled in a 2.0 mL Eppendorf tube. If the leaves did not fit, a second tube was taken. The tubes with the plant tissue were frozen with liquid nitrogen in an isolated cylinder. Where after they were stored in a -80 °C freezer and ultimately they will be sequenced by Detlef Weigel in Cologne (Germany).

Progeny	ms Cvi-0	F1 hybrid (Col x Ler) quartet-1
A	A	A
B	B	B
H	A	B
H	B	A

Figure 4 Scheme used for the reconstruction of the progeny of the cross between male sterile Cvi-0 and the F1 hybrid (Col x L*er*) in the *quartet*-1 background to score the F1 hybrid.

Results and Conclusion

Recombination in *Arabidopsis*

Number of crossovers

In order to obtain a detailed view of crossover formation in *Arabidopsis*, pollen quartets of a F1 hybrid (Col x L*er*) were crossed to a tester line (a male sterile Cvi). Following 253 crosses of individual tetrads to single flowers (see M&M), 36 sets of four (heterozygous) offspring were obtained (i.e. 36 sets of four offspring resulting from one meiosis) that were genotyped using SNP markers. This allowed us to establish the genotypes of the F1 gametes that gave rise to the individual offspring (see figure 5 and M&M for method). Crossover data have been summarized in table 1. On average 9.44 crossovers with a standard deviation of 1.50 per meiosis were observed with a range of 7 to 13 crossovers per meiosis. No significant differences were observed between the number of crossovers on the chromosomes, where 2.39, 1.53, 1.89, 1.33 and 2.31 crossovers per chromosome were observed. On the short arm (the arm of the chromosome with the least base pairs) of chromosome 1, the average number of crossovers was similar compared to the long arm (1.22 for the long arm and 1.17 for the short arm). This is also true for the chromosomes 3 and 5. Chromosome 2 and 4 showed differences between the short and long arm. 0.28 and 0.11 crossovers occurred on average at the short arm of chromosome 2 and 4 respectively. The length in base pairs of the short arms of these chromosomes could explain the lower number of crossovers at these arms. Chromosome 2 and 4 were submetacentric, so the centromere lay closer to one of the telomeres compared with the other centromere. The length of the chromosome of the chromosome arms determines the number of crossovers.

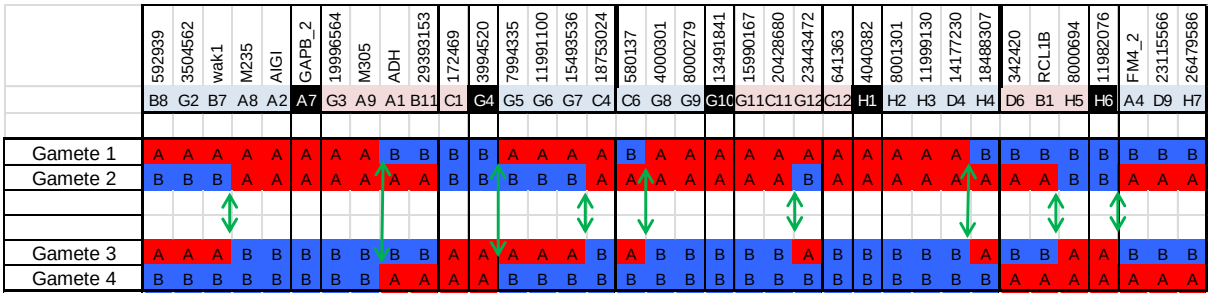

Figure 5 The genotype of four gametes derived from one meiosis. A total number of 9 crossovers occurred in this meiosis. On chromosome 4 only 1 crossover has occurred. The other chromosomes have 2 crossovers. Black numbers refer to the markers most close to the centromere.

Table 1 Crossover numbers in 36 meiosis of an *Arabidopsis* hybrid (Columbia x Landsberg *erecta*) with standard deviation included.

	Number of	Number of Crossovers			Average		
	meiosis	Short arm	Long arm	Total	Short arm	Long arm	Total
Chromosome 1	36	42	44	86	1.17 ± 0.44	1.22 ± 0.48	2.39 ± 0.54
Chromosome 2	36	10	45	55	0.28 ± 0.45	1.25 ± 0.55	1.53 ± 0.50
Chromosome 3	36	32	36	68	0.89 ± 0.46	1.00 ± 0.41	1.89 ± 0.66
Chromosome 4	36	4	44	48	0.11 ± 0.31	1.22 ± 0.42	1.33 ± 0.47
Chromosome 5	36	36	47	83	1.00 ± 0.47	1.31 ± 0.57	2.31 ± 0.66
Total	36	124	216	340	3.44 ± 0.93	6.00 ± 1.03	9.44 ± 1.50

Normalized crossover frequency

Above was shown that chromosomes differ in their number of crossovers, and that this is apparently related to the actual length of the chromosomes. Short chromosome arms (in numbers of bp) have fewer crossovers than long chromosome arms. Perhaps the best way of assessing whether crossovers are evenly distributed over the chromosomes is making a comparison between the physical map (i.e. the placement of markers on the chromosomes in base pairs) and the genetic map (a map indicating the genetic distance between markers in cM (i.e. a measure of the number of crossovers along the chromosome)). Therefore the normalized crossover frequency is calculated by the number of crossovers standardized by the physical length of the genome.

The genetic map was calculated by making use of the recombination patterns of the haploid sets. The genetic distance in centimorgans for the SNP markers was calculated by linkage analysis of the software program Joinmap 4. In **the supplementing data**, the genetic map is depicted against the physical map.

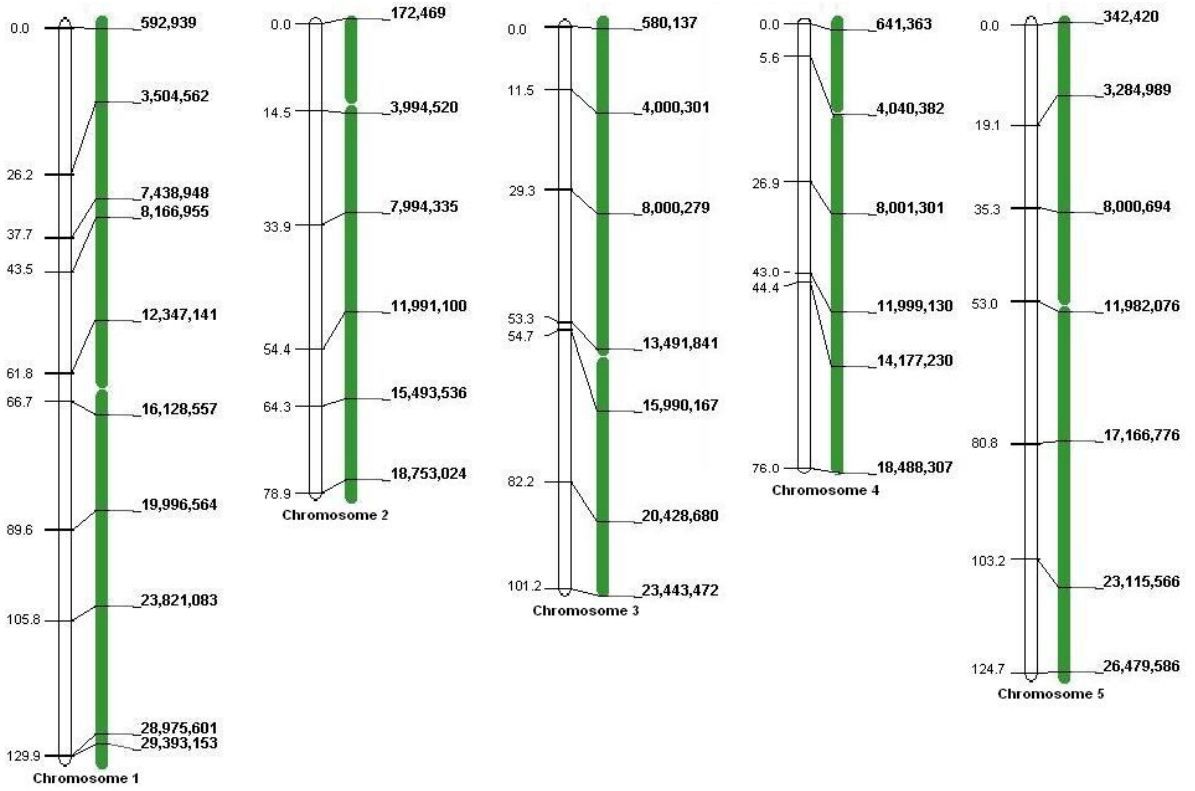

Figure 6 The genetic map (white bars) of *Arabidopsis* thaliana, based on the recombination pattern of F1 hybrid, Columbia and Landsberg *erecta* (Joinmap 4). The marker positions are expressed in cM. Besides the physical map is shown (green bar) where the marker position is expressed in base pairs.

Comparing the genetic map with the physical map, the normalized crossover frequency could be observed. The normalized crossover frequency of the whole genome was one crossover per 350 kb. The intervals with lower crossover frequency could be found in figure 6 (i.e. the distance between the markers at the genetic map (white bars) were much closer related compared with the physical map (green bars)). Three intervals with lower crossover frequencies were observed. These were between SNP marker at 12,347,141 base pairs and the centromere marker at 16,128,557 base pairs at chromosome 1 (1), between the SNP marker at 15,990,167 base pairs and the centromere marker at 13,491,841 base pairs on chromosome 3 (2) and between the SNP marker at 11,999,130 and 14,177,230 base pairs on chromosome 4 (3). The

intervals with lower crossover frequencies lay close to the centromere. Only on chromosome 4, this interval laid further away from the centromere (at chromosome 4, the Col x L*er* inversion is present, where no crossovers occurs, but the observed interval is not in the neighbourhood of this inversion. This inversion can be found on the short chromosome arm (Drouaud 2006)). Intervals with higher crossover frequencies were also observed in figure 7. These intervals were at chromosome 1 (between marker 592,939 and marker 3,504,562), chromosome 3 (between marker 15,990,167 and marker 20,428,680), and chromosome 4 (between marker 14,177,230 and marker 18,488,307). These intervals lay at the end of the chromosome arms. The recombination was suppressed at the centromeres of chromosome 1 and 3 and was higher at the telomeres of chromosome 1, 3 and 4.

Recombination

Near-Reverse Breeding allows the fixation of unknown heterozygous plants by producing complementing homozygous lines for it with the use of Second Division Restitution. Diploid gametes are produced, which were generated to SDR-0 plants. These plants are homozygous at the centromeres. But these SDR-0 plants were also heterozygous at the distal ends due to the occurrence of crossovers. As previously shown, the 36 sets of four offspring resulting from one meiosis present a lot of information about meiosis. These sets made it possible to reconstruct SDR gametes by combining the two haploids, which scored the same for the centromeres. A F2 population was produced by self-pollination of the same hybrid. The amount of homozygosity of the SDR-gametes and F2 population was calculated and compared in the following.

The amount of homozygosity of the SDR-0 plants and the F2 population was calculated using the data of the SNP analysis. Homozygosity is given in estimated absolute numbers of base pairs and not in number of homozygous markers, see **supplementary data** for the calculation. This is calculated for the whole genome and per single chromosome (depicted in table 2). The F2 populations were on average 49% homozygous. The reconstructed SDR gametes were for 53% homozygous, which was only 4% higher than the F2 population. The SDR-0 plants are thus no more homozygous than F2 plants.

Complementing SDR-0 plants are selected in Near-Reverse Breeding (i.e. the plants are selected for being complementary at the centromeres). The SDR-0 plants reconstructed of the 36 sets of four offspring resulting from one meiosis were checked for their complementarity. Heterozygosity was fixed on average on 38% of the genomes of these complementing lines to reproduce the hybrid. So in 62% of the genome could still segregate. On average 0.4% of the genome of the complementing SDR-0 plants was not capable to reconstruct heterozygosity. This is also done for the F2 population were many F2 plants were selected for their complementarity. A selection was necessary to check the possibility to reproduce the starting hybrid. The selected complementary F2 plants could fix 15% of heterozygosity and for 4% of the genome, the heterozygosity was lost. The SDR-0 plants give a higher advantage than the F2 plants for selecting complementing plants and reconstruct the starting heterozygous plant.

Table 2 The recombination in gametes in percentage for the whole gamete and for only the chromosomes. Besides, the amount of homozygosity in simulated SDRs and FDRs derived from the four products of a single meiosis is depicted.

	Chromosome 1	Chromosome 2	Chromosome 3	Chromosome 4	Chromosome 5	Total
Homozygosity:						
F2 population (2n)	47.57%	49.43%	50.32%	49.79%	49.32%	49.16%
SDR (2n)						
From same meiosis	50.62%	53.63%	58.35%	54.62%	51.16%	53.39%
FDR (2n)						
Maximum	41.32%	43.83%	35.42%	43.36%	37.72%	40.07%
Minimum	8.07%	2.54%	6.23%	2.02%	11.12%	6.54%

Besides SDR gametes also FDR gametes could be reconstructed. The FDRs could only be reconstructed per chromosome and not per gamete, because of the chromatid orientation which occurred in anaphase II, whereby the amount of homozygosity was hard to give. The homozygosity was calculated the same way as for the SDR-0 plants and the F2 population. In the case that all chromosomes with the highest homozygosity were pooled together in the FDR gamete, the amount of homozygosity was 40.07%. If the chromosomes with the lowest homozygosity were pooled together; the amount of homozygosity was 6.54%. So the use of FDR gametes for reproducing the heterozygous plant gave no advantage over the use of SDR gametes or the F2 population.

Producing haploids

Haploid plants of the F1 hybrid (Col x L*er*) in the *quartet*-1 background were produced by crossing this hybrid with the CENH3. When crosses are made with the CENH3 inducer, most resulting seeds do not contain an embryo. We therefore set out to see whether embryo rescue could generate higher levels of offspring. Both embryo rescue as mature seeds were used for generating full grown plants. 240 siliques (240 flowers were pollinated with one pollen quartet) were harvested for embryo rescue after 12 days of pollination. We recovered a total of 674 embryos, of which 340 embryos were transferred to the medium. The other embryos were shrivelled, did not get a well-developed embryo or died. The embryos grew on culture, where some embryos died. Finally 247 embryos could be genotyped. Crosses between the F1 hybrid in *quartet*-1 background and the CENH3 produced haploids, diploids and even self-pollinations of the CENH3. The results of the genotyping are depicted in Table 3. In the case of the mature seeds, the seeds were sown and have developed to full grown plants. They were genotyped, but only for the presence of the CENH3 rescue insert (PCR for GFP); hereby diploids and self-pollinations could not be distinguished from each other (see table 3). Results are shown in Table 3. The fraction of haploids in both cases (embryo rescue and seed-derived haploids) was 15% of the plants.

Table 3 The results of the crossings between CENH3 and the F1 hybrid (Col x Ler) in the *quartet*-1 background, where haploids, diploids and self-pollinations of the CENH3 are produced.

Number of individuals	Embryo Rescue		Seeds	
	Number	Percentage	Number	Percentage
Self-pollinations	194	78.54%	115	83.94%
Diploid	15	6.07%		
Haploid	38	15.38%	22	16.06%

It was not possible to genotype more than two haploids of a single meiosis. To generate information from a single meiosis, in total 253 crosses between the F1 hybrid (Col x L*er*) in the *quartet*-1 background and the male sterile Cvi-0 were made. In Figure 7, for both as CENH3 as male sterile Cvi-0 the number of seeds obtained from the F1 hybrid in the *quartet*-1 mutant were illustrated in percentage. 3 or 4 seeds were obtained in 45% of the cases and from these seeds meiotic information was generated. The percentage of pollen tetrads producing 4 seeds was 13%. The male sterile produced more seeds from the cross, whereby the seeds which were obtained were all coming from the pollen tetrad and were no self-pollination, which occurred at the CENH3.

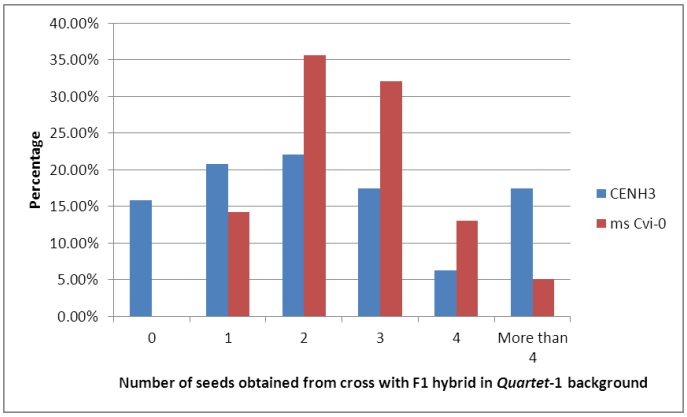

Figure 7 The number of seeds (in percentage) obtained from the crosses with the F1 hybrid in *quartet*-1 background.

SDR-0 gametes by N_2O treatment

<u>N_2O treatment</u>

To test whether the induction of 2n (and preferably SDR-) pollen is possible in *Arabidopsis* (and hence whether Near-Reverse Breeding is possible), we attempted to induce 2n pollen using nitrous oxide gas under high pressure. In crops like lily and tulip, N_2O treatment is a documented used tool to induce 2n pollen for increasing the ploidy level, and allowing interspecific crosses. Tulip and lily undergo N_2O treatment at 6 bars in a pressure cylinder for 24 hours when flower buds are in meiosis (Barba-Gonzalez 2006; Akutsu 2007). For *Arabidopsis*, the same assumptions were used, whereby the pressure was increased and decreased to 6 bar in 1.5 hours. The *Arabidopsis* plants treated with N_2O under the above described conditions came with wilted flower buds out of the treatment. Three or four days after treatment, the plants died, although sometimes the plants recovered. In this case, the flowers were unable to produce normal pollen. The pollen tetrads fell apart and dead pollen were present in the tetrads. Also the amount of pollen was less compared with untreated pollen. So the treated *Arabidopsis* plants in the *quartet*-1 background produce aberrant pollen tetrads. This also applied for the *Arabidopsis* plants which were not in the *quartet*-1 background. We there observed the same phenomena: Irregular pollen, with high levels of pollen abortion.

The induction of irregular pollen, and the frequent dying of treated plants following N_2O treatment has not been reported in Lilies or tulips and under the used conditions, lilies and tulips survive much better (Jaap van Tuyl, pers. comment, 2010). We set out to change the conditions such that we would reduce mortality in the treated plants, and increase pollen viability. The starting conditions were changed to produce viable pollen and plants, which survive the treatment. Sander Pot (pers. comment 2010) pointed to the possible problems during decompression as the cause of dead *Arabidopsis* plants or unviable pollen. The ideal gas law of Boyle ($\rho \times V$ = constant) will explain this, and is illustrated in figure 8. When the gas (N_2O) pressure (ρ) decreases, the volume (V)

will automatically increase, since the product of p * V is to remain constant. So following a decrease in pressure, the gas volume will increase, possibly damaging plant tissues by causing air bubbles to form in tissues, cells and vessels. It can be seen in the graph that especially the decrease from 2 to 1 bar in pressure will cause a twofold increase of the gas volume in the plant, making the last stages of decompression the most crucial. We therefore decided to vent the N_2O much slower. The time of decompression of N_2O was changed from 1.5 to 8 hours. The *Arabidopsis* plants had no wilted flowers after the treatment with the corrected setpoints. Plants were capable to produce higher amounts of pollen as compared with the previous experiments after five, six and seven days. The pollen size was measured in pixels with ImageJ after making photos of the pollen. After seven days of treatment, the following pollen size distribution was made (figure 9, blue bars). A wide range of pollen size range was observed. Two peaks, one between 2,400 and 4,200 pixels and one between 5,400 and 6,800 pixels were shown in figure 9. Only one bar was observed for the untreated (wild type) pollen (red bars). The pollen, which belongs to the second peak of the with nitrous oxide treated pollen were interpreted to be 2n pollen. In figure 11 the pollen after the treatment with N_2O were depicted. These photos were used for pollen size measurements.

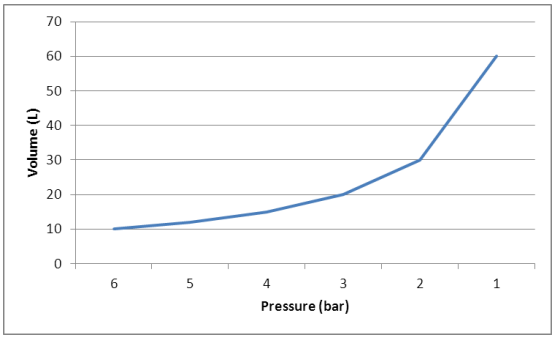

Figure 8 The ideal gas law of Boyle (ρ x V = Constant), used as reference for the venting the pressure cylinder (Pot 2010, pers. comment). Be aware: the volume will increase in twofold if the pressure decreases from 2 to 1 bar. The constant is 60 in this example.

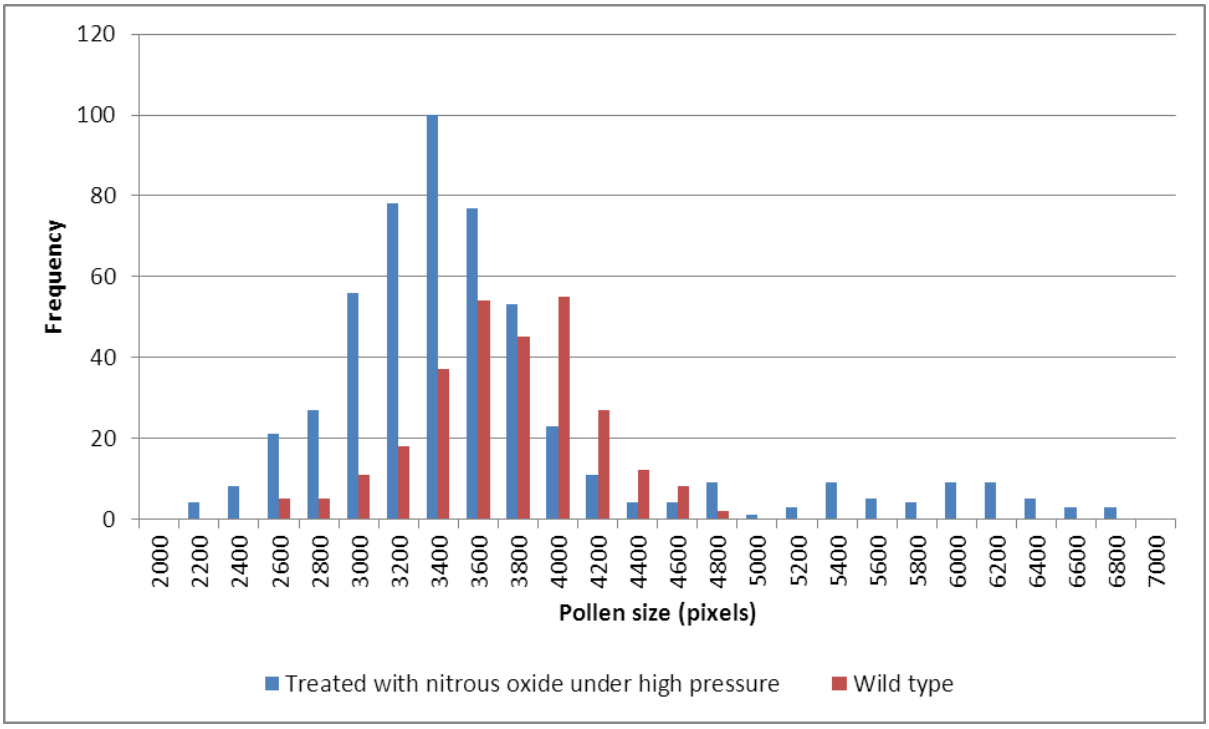

Figure 9 The pollen size distribution of pollen of the F1 hybrid (Columbia x Landsberg *erecta*) treated with nitrous oxide under high pressure and the wild type (untreated) F1 hybrid.

The larger (2n) pollen were transferred to the stigma of the genome eliminator to produce 2n plants (SDR-0 plants), as will be described below.

SDR-0 plants

The 2n pollen produced from the nitrous oxide treatment, were crossed with the haploid inducer to produce seeds. These seeds were sown to check the nitrous oxide indeed produced SDR gametes (2n gametes). Not all the crosses lead to a full grown seed. A lot of seeds were shrivelled. In total 39 seeds were able to germinate and produce a plant. The plants were genotyped, whereby 37 plants were a result of a self-pollination of the CENH3. The other two plants did not show crossovers events. The genotype of these plants can be found in Figure 10. The plants looked more like originating from a reverse breeding population. In Figure 10 also the possible gametes coming from the F1 hybrid Columbia and Landsberg *erecta* has been shown. Plant 36 was a cross, whereby no genome elimination took place by the maternal chromosomes of the CENH3. The paternal chromosomes were a result of chromosome orientation, whereby chromosomes 1, 4 and 5 derived from Landsberg *erecta* and the chromosomes 2 and 3 derived from Columbia. Plant 22 was according to the genotyping an exact genotypic copy of the F1 hybrid. This hybrid could be produced in two different ways. The first possible way was a gamete, where all the Landsberg *erecta* chromosomes were inherited from the F1 hybrid. After fusion of the zygote, the maternal genotype was not eliminated, whereby the Columbia background came from the haploid inducer. The second possibility is that a FDR gamete (without crossovers) was crossed to the CENH3 inducer, whereby the gamete was an exact copy of the paternal F1 hybrid. In this case genome elimination took place with the maternal CENH3. No SDR-0 plants were produced, but possibly the nitrous oxide treatment suppress crossovers.

	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
22	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
gamete 1	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
gamete 2	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
36	H		H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	H	H	H
gamete	B		B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B

Figure 10 The genotypes of two plants (22 and 36) obtained from a cross between F1 hybrid treated with nitrous oxide under pressure and CENH3. For each plant, the possible gametes coming from the paternal F1 hybrid are depicted.

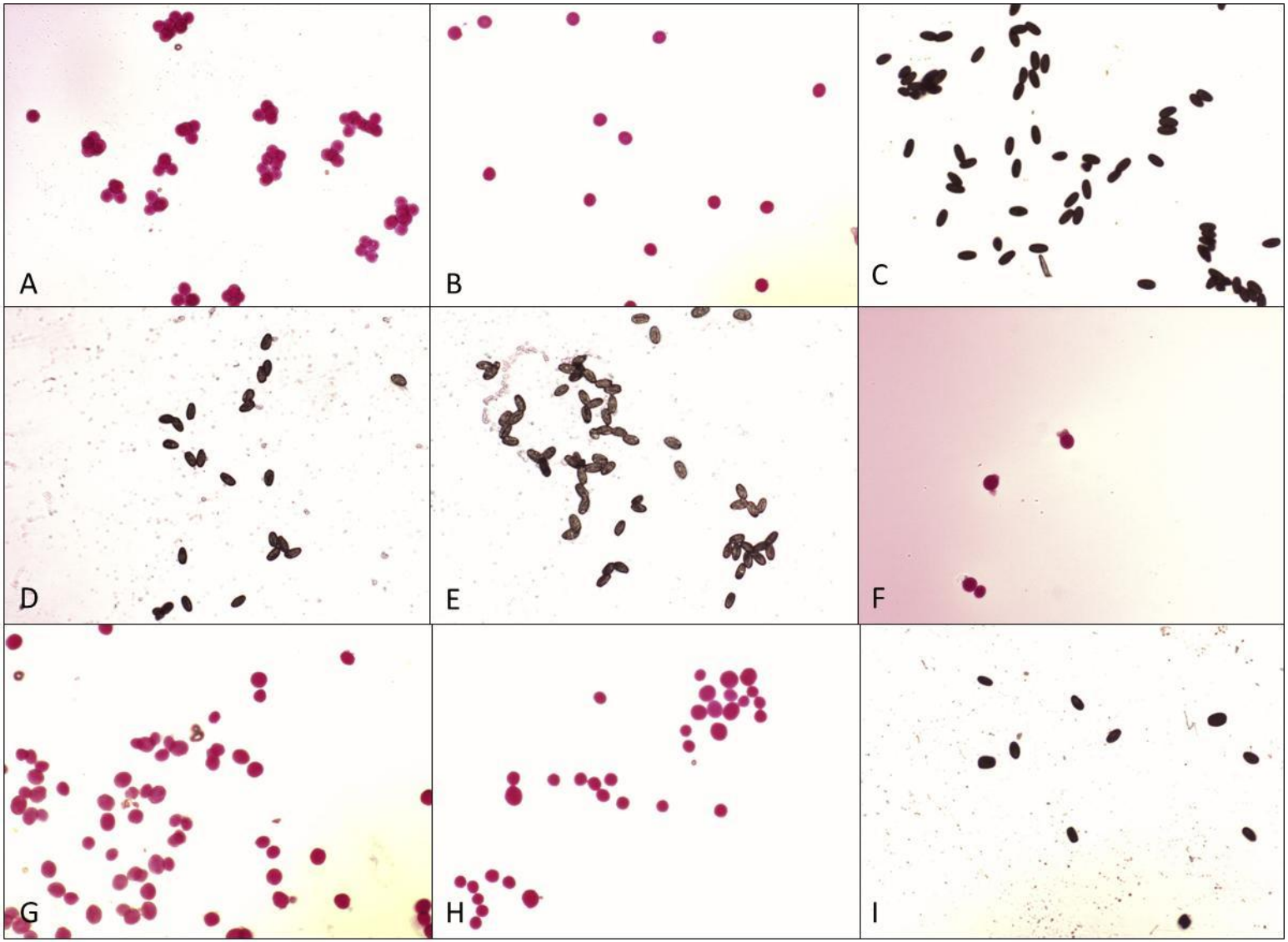

Figure 11 Nine pictures of pollen from F1 hybrid Columbia x Landsberg *erecta*. (A), (B) and (C) are untreated pollen, whereby in (A) the pollen are in the *quartet-1* background. (D), (E) and (F) are pollen of the hybrid in the *quartet*-1 background treated with N_2O with a small vent time, whereby the pollen tetrad structure is fallen apart. (G), (H) and (I) are pollen of a wild type hybrid treated with N_2O with a longer vent time, whereby 2n pollen are produced. These are the bigger pollen in the picture. The pollen in the pictures (A), (B), (F), (G) and (H) are all treated with lactophenolacid fuchsin, so the pollen could be observed easier.

Improving Near-Reverse breeding: the need for crossover control

Homozygous lines are produced in Near-Reverse Breeding with the use of SDR gametes. Complementing homozygous lines are selected to produce doubled haploids and reconstruct the starting hybrid with complementing doubled haploids. Complementing homozygous lines fix a specific amount of heterozygosity. Below, it is discussed to get the fixed heterozygosity in the complementing SDR-0 plants as much as possible making use of the results and assumptions.

If random haploids were taken to produce the F2 population and also a real F2 populations of the F1 hybrid (Col x L*er*) were produced, both showed to be roughly 50% heterozygous, concurrent with theoretical expectations: With the formula given by Allendorf (2006), this amount of recombination was the same, namely $(1/2)^{(2-1)}$ = 50%. So 50% of the F2 was homozygous. The amount of homozygosity for SDR gametes was estimated on 60% in according to Carputo (2003). But by simulating SDR gametes from the four haploids derived from the same meiosis, the amount of homozygosity of 36 SDR gametes was 53% (virtually similar to an F2 population).

Although not having higher levels of homozygosity than a F2 plant, SDR-plants would nevertheless have a great advantage: two SDR-0 plants can be selected for which the centromere areas of the two are complementing. One should regard such a set of the complementing SDR-0 plants as the starting point for further breeding: by generating DH's from the SDR-0 plants, the remaining levels of heterozygosity on the chromosome ends could be removed, and one should choose the generated DHs such that the DHs together preserve as much of the alleles that were present in the starting heterozygote. If the SDR-0 plants, which were coming from the 36 set of four offspring of one meiosis, were selected for their complementarity, already 38% of the heterozygosity was fixed, where we expected an amount of 33% (see **supplementary data** for calculation).

The starting heterozygous plant was also reconstructed with F2 plants. In *Arabidopsis*, the F2 population could fixate 15% of its heterozygous alleles by looking for complementing areas. A strong selection method was necessary to reconstruct as much as possible of the starting hybrid.

It was discussed that the level of homozygosity in SDR-0 plants can be increased when crossover levels are reduced. When crossovers are reduced from 2 to one crossover per chromosome, the levels of homozygosity in SDR-0 plans would immediately rise to 75%. Following that same rationale, decreasing crossover frequencies to levels of 1 crossover per two chromosome pairs would. In table 4, the amount of homozygosity is depicted for several crossover frequencies.

Firstly Near-Reverse Breeding will be discussed by a theoretical approach, whereby the effect of crossover reduction is explained. Crossovers can occur randomly on the chromosomes, complicating a theoretical analysis. We therefore assume the presence of one crossover per chromosome arm (as usual in *Arabidopsis*) and assume it to be always present at the same position.

For convenience the Near-Reverse Breeding is discussed for only one chromosome arm. In figure 12, the starting heterozygous chromosome arm is shown at the top (the black bars are the centromeres) **(a)**. The arms were duplicated just before meiosis and one crossover occurs at the green bar in meiosis I **(b)**. The second meiotic division is omitted, so SDR gametes are produced, which are generated to produce SDR-0 plants **(c)**. For one chromosome arm, only two possible SDR-0 plants were possible (as showed in figure 12). Doubled haploids are produced of these SDR-0 plants. 2 different doubled haploids can be produced for each SDR-0 plant **(d)**. Complementing doubled haploids are selected (i.e. complementing for the centromeres) and crossed with each other. The four possible results of these crossings are depicted in figure 7 **(e)**. In 2 of the 4 cases, the heterozygosity of the starting plant is maintained **(a)**. This chance to retain complete heterozygosity on this chromosome arm is ½. For *Arabidopsis*, which contains 10 chromosome arms, the chance to reproduce the hybrid equals $(1/2)^n$ (where n is the number of chromosome arms) = $(1/2)^{10}$ = 1/1,024 (0.00097). So the chance to perfectly reproduce a tomato hybrid can be calculated assuming the above mentioned standard. Tomato contains 12 chromosomes, so the chance equals $(1/2)^{24}$ = 1/16,777,216 (= 5.96×10^{-8}).

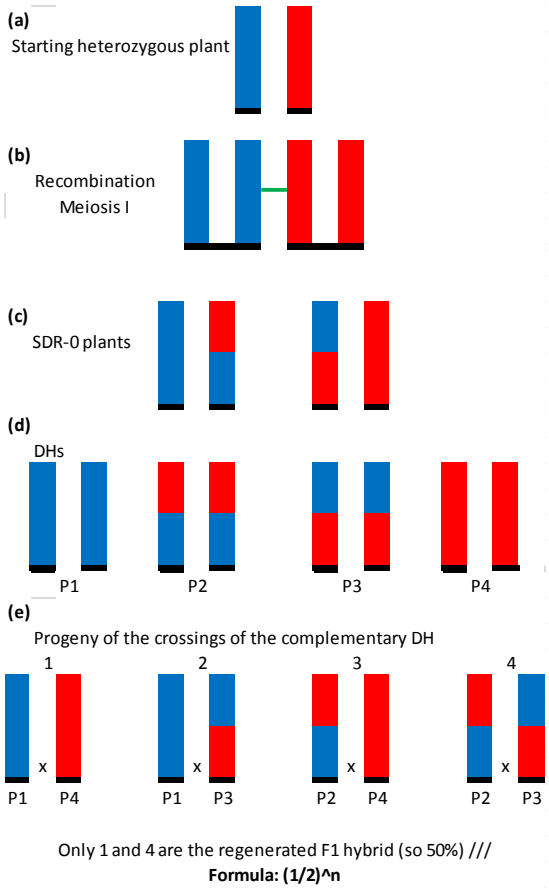

Figure 12 The starting heterozygous plant is depicted for one chromosome arm. This chromosome arm is reconstructed by the use of Near-Reverse Breeding. For more details see text on the left.

Near-Reverse Breeding with reduced crossovers

The chance to reproduce the starting heterozygous plant in *Arabidopsis* is small and will only get smaller if the chromosome number increases as seen in the example for tomato. We propose to reduce the crossover frequency and observed what this meant for the reproduction of the starting hybrid (see also table 4). The crossovers were reduced with 50% for this approach. In figure 13, we explain this effect schematically. We start again with a heterozygous chromosome arm **(a)**. Crossovers occur at prophase I, but only in 50% of the cases crossovers are formed. **(b1)** shows the 50% of the cases a crossover occurs and **(b2)** represents the 50% with no crossovers. Therefore four possible SDR-0 plants are produced, two with crossovers **(c1)** and two without crossovers **(c2)**. Again doubled haploids are produced of these SDR-0 plants **(d)**. Doubled haploids, complementing for the centromeres are crossed with each other **(e)** to reproduce the starting heterozygous plant **(a)**.

In total, 16 crosses between complementing doubled haploids were made and depicted in figure 13. For instead doubled haploid 1 **(d)** is crossed with the doubled haploids 3, 4, 7 and 8, which are complementing at the centromeres. In cross between 1 and 3, the progeny shows homozygosity at the distal ends, so the starting hybrid is not

regenerated. For the crosses between 1 with 4, 7 and 8, the heterozygous plant **(a)** is perfectly reproduced. In 6 of the 16 cases, the hybrid is not regenerated, but in the other 10 cases the hybrid is regenerated (see figure 8). The chance to maintain the heterozygosity for one chromosome arm is 10/16 = 5/8 = 62.5%, which is higher compared with no crossover reduction.

If an organism has 10 chromosome arms, just like *Arabidopsis*. The chance is $(5/8)^{10}$ = 1/110 = 0.0091. The chance to reproduce the heterozygous plant is increased almost 9 times in *Arabidopsis* compared with no crossover reduction. The formula which we draw for the chance to reproduce the starting hybrid with 50% reduction of crossovers equals $(5/8)^{n}$, where n again stands for the number of chromosome arms. If we use this formula for tomato, the chance is $(5/8)^{24}$ = 1/79,228 (= 1.26×10^{-5}) to reproduce the hybrid. This is still small, but the chance increases about 200 times compared with no crossover reduction. So the chance to reproduce the starting heterozygous plant could be increased by crossover reduction. If the crossovers will be reduced with a higher percentage, the chance will increase more and more.

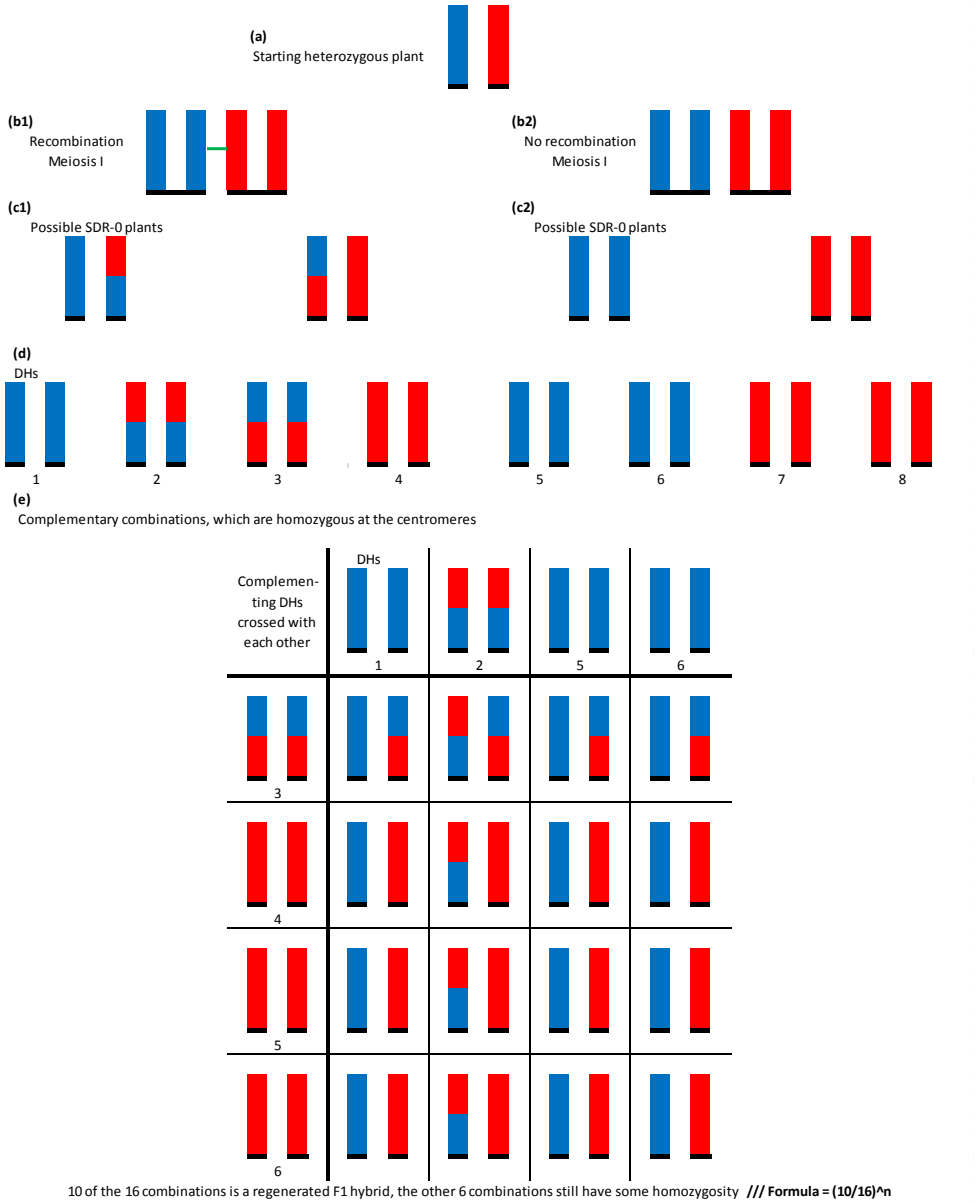

Figure 13 The starting hybrid is depicted for one chromosome arm. This heterozygous plant is reconstructed by Near-Reverse Breeding, whereby the crossovers are reduced with 50%. The chance to reproduce the hybrid is shown schematically. For more details see text.

The amount of homozygosity is higher in SDR-0 gametes where the crossovers frequency is lower (shown in table 4). Besides the theoretical approach describes the higher chance to reproduce the starting heterozygous plant. Table 4 shows the amount of the fixed heterozygosity of the genome, if two complementing SDR-0 plants were selected (for more details see **supplementary data**). If the number of crossovers is reduced with a higher amount, the homozygosity of the SDR-0 will increase as well as the percentage of fixed heterozygosity. Figure 14 will underline this. Hereby the chance to reproduce the starting hybrid will increase.

Table 4 The amount of homozygosity in SDR-0 plants and the amount of fixed heterozygosity in two complementing SDR-0 plants at the numbers crossovers per chromosome.

Crossovers per arm	Crossovers per chromosoom	Homozygosity in SDR-0	Percentage fixed heterozygosity in two complementing SDR-0
1.00	2.00	50.00	33.33
0.50	1.00	75.00	58.33
0.25	0.50	87.50	77.08
0.13	0.25	93.75	88.02
0.00	0.00	100.00	100.00

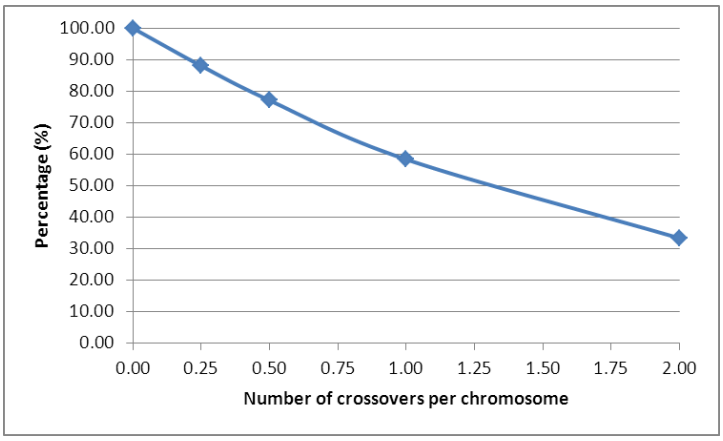

Figure 14 The amount of fixed heterozygosity in a set of two complementing SDR-0 plants (vertical) as a function of the number of crossovers per chromosome. Note that with lower numbers of crossovers, the amount of fixed heterozygosity increases.

Near-Reverse Breeding becomes more interesting and powerful breeding technique, if on combination could be found between partial suppression of the crossovers with the production of SDR gametes. This will be explained with the use of an example in tomato. Reverse Breeding is almost impossible in tomato, where the chance of a balanced gamete is $(1/2)^{12}$ = 1 / 4,096 (Dirks 2009), where tomato contains 12 chromosomes. In Near-Reverse Breeding, 50% of the SDR gamete in tomato is homozygous. Besides table 4 shows only 33% of heterozygosity, which is fixed if two complementing SDR-0 plants were selected. Near-Reverse Breeding should become more feasible if crossovers could be reduced with for instance 75%. In this case, 0.5 crossovers per chromosome occur instead of 2 crossovers per chromosome. 77% of the heterozygosity is fixed in one step, whereby the chance of a balanced gamete is $(1/2)^{6}$ = 1 / 64 (Dirks 2009) (there are 6 univalent pairs segregation).

Reconstruction of heterozygous plants with Reverse Breeding is unlikely in tomato, but with Near-Reverse Breeding with suppression of crossovers, this should become more feasible.

Discussion

Near-Reverse Breeding is a proposed new breeding technique, whereby interesting heterozygous plants can be selected and the breeding lines for that plant can be obtained through the induction of SDR gametes. As was indicated in the last paragraphs of the results section, we were not able to regenerate SDR gametes as SDR-0 plants. Nevertheless, the data obtained give a wealth of information on meiotic recombination and have some profound implications for the further development of Near-Reverse Breeding as a breeding tool. A good understanding of meiotic recombination is of the highest importance to understand the usefulness of Near-Reverse Breeding. Therefore the use of the *quartet*-1 mutant proved indispensable. The use of the F1 hybrid in the *quartet*-1 background made it possible to generate the four gametes which were result of one single meiosis. These four gametes were crossed to a male sterile Cvi-0 plant. The genotype of the four gametes could be inferred after genotyping with 36 markers that were evenly spaced over the whole genome. This allowed the reconstruction of segregation events in both meiosis I as meiosis II. The experiments described here provide a good starting point for further research and give various clear clues on the best approaches to develop Near-Reverse breeding as a powerful breeding tool. The main insights will be discussed below.

Looking to the recombination, 36 meiosis have been analysed. The F1 hybrid (Col x L*er*) had on average 9.44 crossovers per meiosis, which is in accordance with data of Copenhaver (1998) where they found 9.0 crossovers in the same F1 hybrid. Sanchez-Moran (2002) observed 9.1 and 8.7 crossovers per meiosis for the two parental ecotypes Columbia and Landsberg respectively. On average one crossover occurred on every chromosome arm, which was also observed at Copenhaver (2002) and Sanchez-Moran (2002). We observed fewer crossovers to occur on the short arms of chromosomes 2 and 4 (0.28 and 0.11 respectively). These chromosomes were submetacentric, which means the centromere was nearly located to one of the telomeres. The lower crossovers on the short chromosome arms show a direct correlation between the chromosome length and the number of crossovers (Copenhaver 1998; Sanchez-Moran 2002, Drouaud 2006). A genetic map could be calculated using the recombination patterns. In the table below, the genetic map composed by different researches, namely AGI and Classical map (TAIR 2010) and Erik Wijnker (2010 pers. comment) is shown. The results of this study were comparable with the others. Only 36 meiosis have been analysed for this study, where more meiosis could be observed by using the crosses where 3 seeds were obtained from.

Table 5 The genetic map of *Arabidopsis thaliana* in cM composed by different researches (TAIR 2010; Wijnker 2010, pers. comment).

	Research			
Chromosome	AGI	Classical map	Erik Wijnker	Own
1	135.0	122.0	139.4	129.9
2	97.0	77.0	90.7	78.9
3	101.0	96.0	110.7	101.2
4	125.0	76.0	83.0	76.0
5	139.0	98.0	120.7	124.7
Total	597.0	469.0	544.5	510.7

The 36 sets of four offspring resulting from one meiosis give information about the meiosis so SDR gametes and therefore SDR-0 plants could be reconstructed. These plants showed 53% homozygosity, which is equal to a F2 population. Nevertheless SDR-0 plants have an advantage over the F2 plants to reconstruct the starting hybrid, because of their complementarity. If complementing SDR-0 plants were selected, 38% heterozygosity was already fixed, where 33% was expected. The F2 population showed 15% of heterozygosity, which was fixed, after selecting the most complementing lines. Complementing SDR-0 plants could reproduce the starting hybrid easier compared with F2 plants.

The 33% heterozygosity, which is fixed in complementing SDR-0 plants, is not that high. This means that still 67% of the genome could segregate. Crossover reduction was proposed to fix the amount of heterozygosity. If 50% of the crossovers were reduced (i.e. normally one crossover per chromosome arm occurs, but now one crossover per two chromosome arms occurs), the amount of homozygosity increases in the SDR-0 plants. By selecting complementing SDR-0 plants could fix now 58% of the heterozygosity. Lower crossover frequencies increase the degree of heterozygosity fixation in complementing pairs.

Before complementing SDR-0 was selected for reconstruction of the starting hybrid, SDR gametes and SDR-0 plants have to be induced. Nitrous oxide under high pressure was used to produce SDR gametes. Larger pollen were observed after the treatment, which could be interpreted as 2n gametes. These 2n gametes were generated to plants, whereby only two plants were a result of the treatment. No SDR-0 plants were observed, but in this case, the plants do not show crossovers. Probably the plants are affected only in meiosis I, where the crossovers are formed. The nitrous oxide under high pressure seems to suppress also the crossover formation.

Only two plants were produced, which are a result of the nitrous oxide treatment. The low number of generated plants could be a result of the haploid inducer, which did produce a low amount of seeds due to improper elimination of the genome of the haploid inducer (Chan 2010; Marimuthu 2011). The large range of pollen size observed after the nitrous oxide treatment could be explained by the absence of crossovers. This leads in many cases to missegregation of the chromosomes, leading to aneuploid gametes (Mézard 2007). The low frequency of generated plants of the N_2O treatment could be explained by the haploid inducer and the missegregation of chromosomes due to the absence of crossovers. The reduction of crossovers was not observed in further research.

Conclusion

Near-Reverse Breeding could be a useful approach to reproduce the heterozygous plant with two complementing homozygous lines with the use of SDR gametes. Crossover reduction could increase the degree of heterozygosity fixation in complementing pairs. The nitrous oxide under pressure treatment did not produce SDR gametes, whereby possibly not the right meiotic stage was affected. Further researches should demonstrate the success of Near-Reverse Breeding:

- Proof of concept of Near-Reverse Breeding is important and could be demonstrated in *Arabidopsis*, where SDR gametes / SDR-0 plants are produced.
- The nitrous oxide treatment should be researched to produce both as SDR gametes as gametes with crossover suppression. Meiosis I is probably affected in the case of crossover suppression. For the production of SDR gametes, the meiosis II should be affected. Whereby the right stage of meiosis II should be determined. A possible other research could be to suppress crossovers in combination with SDR induction. This can be done by two treat the plant twice with nitrous oxide under pressure. So meiosis I and II should be affected.

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References

Akutsu, M., S. Kitamura, et al. (2007). "Production of 2n pollen of Asiatic hybrid lilies by nitrous oxide treatment." Euphytica **155**(1): 143-152.

Allendorf, F. W. L., G. (2006). Conservation and the Genetics of Populations, Wiley-Blackwell.

Barba-Gonzalez, R., C. Miller, et al. (2006). "Nitrous oxide (N2O) induces 2 gametes in sterile F1 hybrids between Oriental × Asiatic lily (Lilium) hybrids and leads to intergenomic recombination." Euphytica **148**(3): 303-309.

Baudat, F. and B. de Massy (2007). "Regulating double-stranded DNA break repair towards crossover or non-crossover during mammalian meiosis." Chromosome Research **15**(5): 565-577.

Beal, W. J. (1879). Experiments & other work of the Horticultural depart. Michigan, Michigan State College of Argiculture and Applied Science.

Bennett, M. D., I. J. Leitch, et al. (2003). "Comparisons with Caenorhabditis (100 Mb) and Drosophila (175 Mb) Using Flow Cytometry Show Genome Size in *Arabidopsis* to be 157 Mb and thus 25 % Larger than the *Arabidopsis* Genome Initiative Estimate of 125 Mb." Annals of Botany **91**(5): 547-557.

Carputo, D., L. Frusciante, et al. (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.

Chan, S. W. L. (2010). "Chromosome engineering: power tools for plant genetics." Trends in Biotechnology **28**(12): 605-610.

Cole, F., S. Keeney, et al. (2010). "Evolutionary conservation of meiotic DSB proteins: more than just Spo11." Genes & Development **24**(12): 1201-1207.

Copenhaver, G. P., W. E. Browne, et al. (1998). "Assaying genome-wide recombination and centromere functions with *Arabidopsis* tetrads." Proceedings of the National Academy of Sciences of the United States of America **95**(1): 247-252.

Darlington, C. D. (1939). The Evolution of Genetic Systems. London, John Innés Horticulture institute.

Darwin, C. (1876). The effects of cross and self fertilisation in the vegetable kingdom. London, John Murray.

Dirks, R. (2003). Reverse Breeding. Rijk Zwaan Zaadteelt en Zaadhandel B.V. The Netherlands. **WO/2003/017753:** 47.

Dirks, R., K. Van Dun, et al. (2009). "Reverse breeding: a novel breeding approach based on engineered meiosis." Plant Biotechnology Journal **7**(9): 837-845.

Drouaud, J., C. Camilleri, et al. (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.

Dun, C. M. P. v. (2008). Near-Reverse Breeding. United States Patent Application 20080098496. USA. **20080098496:** 26.

Francis, K. E., S. Y. Lam, et al. (2006). "Separation of *Arabidopsis* Pollen Tetrads Is Regulated by QUARTET1, a Pectin Methylesterase Gene." Plant Physiology **142**(3): 1004-1013.

Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*." Proceedings of the National Academy of Sciences **104**(10): 3913-3918.

Hamant, O., H. Ma, et al. (2006). "Genetics of meiotic prhophase I in plants." Annual Review of Plant Biology **57**(1): 267-302.

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

Lichten, M. (2001). "Meiotic recombination: Breaking the genome to save it." Current biology : CB **11**(7): R253-R256.

Mancera, E., R. Bourgon, et al. (2008). "High-resolution mapping of meiotic crossovers and non-crossovers in yeast." Nature **454**(7203): 479-485.

Marimuthu, M. P. A., S. Jolivet, et al. (2011). "Synthetic Clonal Reproduction Through Seeds." Science **331**(6019): 876.
Mézard, C. (2006). "Meiotic recombination hotspots in plants." Biochem. Soc. Trans. **34**(Pt 4): 531-534.
Mézard, C., J. Vignard, et al. (2007). "The road to crossovers: plants have their say." Trends in Genetics **23**(2): 91-99.
O'Connor, C. (2008). Meiosis, genetic recombination and sexual reproduction. Chromosomes and cytogenetics. Boston, Boston College.
Peirson, B. N., H. A. Owen, et al. (1996). "Characterization of three male-sterile mutants of *Arabidopsis* thaliana exhibiting alterations in meiosis." Sexual Plant Reproduction **9**(1): 1-16.
Plug, A. W., J. Xu, et al. (1996). "Presynaptic association of Rad51 protein with selected sites in meiotic chromatin." Proceedings of the National Academy of Sciences **93**(12): 5920-5924.
Pot, S. (2010). Decompression of gas treatments. Wageningen, Bio Science WUR.
Preuss, D., S. Rhee, et al. (1994). "Tetrad analysis possible in *Arabidopsis* with mutation of the QUARTET (QRT) genes." Science **264**(5164): 1458-1460.
Ravi, M. and S. W. L. Chan (2010). "Haploid plants produced by centromere-mediated genome elimination." Nature **464**(7288): 615-618.
Robinson, P. and Dr J. Holme (2011). "KASP version 4.0 SNP Genotyping Manual" KBioscience
Ronceret, A. S., M.J.; Pawlowski, W.P. (2007). Chromosome Dynamics in Meiosis. S.-V. B. H. 2007. Berlin, Cornell University, Ithaca,.
Sanchez-Moran, E., S. J. Armstrong, et al. (2002). "Variation in Chiasma Frequency Among Eight Accessions of *Arabidopsis* thaliana." Genetics **162**(3): 1415-1422.
Shingu, Y., T. Mikawa, et al. (2010). "A DNA-binding surface of SPO11-1, an *Arabidopsis* SPO11 orthologue required for normal meiosis." FEBS Journal **277**(10): 2360-2374.
Shull, G. H. (1908). The composition of field maize, Report of the American Breeders Association 4**:** 296-301.
Sung, P. and H. Klein (2006). "Mechanism of homologous recombination: mediators and helicases take on regulatory functions." **7**(10): 739-750.
Székvölgyi, L. and A. Nicolas (2010). "From meiosis to postmeiotic events: Homologous recombination is obligatory but flexible." FEBS Journal **277**(3): 571-589.
Szostak, J. W., T. L. Orr-Weaver, et al. (1983). "The double-strand-break repair model for recombination." Cell **33**(1): 25-35.
TAIR, T. A. I. C.-. (2010). "About *Arabidopsis*." Retrieved 8 september, 2010, from http://www.*Arabidopsis*.org/portals/education/about*Arabidopsis*.jsp.
Tuyl, J. (2010). Nitrous oxide treatment in lily and tulip. Wageningen, Plant Breeding WUR
Weerenbeck (2009). "(Near) Reverse Breeding)."
Wijeratne, A. J., C. Chen, et al. (2006). "The *Arabidopsis* thaliana PARTING DANCERS Gene Encoding a Novel Protein Is Required for Normal Meiotic Homologous Recombination." Mol. Biol. Cell **17**(3): 1331-1343.
Wijnker, E. (2010). Genetic map vs. physical map of *Arabidopsis thaliana*. Wageningen, Laboratory of Genetics WUR
Xu, S., T. Clark, et al. (2008). "Gene conversion in the rice genome." BMC Genomics **9**(1): 93.

Supplementary Data

Table 6 Overview of used cultivars of *Arabidopsis thaliana* and were they were obtained from.

Accession	stocknumber	Remark
Ler	CS20	
Col	N601000	
Ler (qrt-1 qrt-1)	N8050	
Col (qrt-1 qrt-1)	N660403	
ms Cvi-0		ms mutant from een Cvi-0 ems population
CENH3		Simon Chan, University of Califonial, Davis
GEM		Simon Chan, University of Califonial, Davis
		F1 progeny of cenh3-1/cenh3-1 GFP-tailswap/GFP-tailswap (female) to cenh3-1/cenh3-1 GFPCENH3/GFPCENH3 (male)

Table 7 Used substances for growth mediums and sterilization processes

Product	Company	Product number	Molecular formula	Size	Remark
Murashige & Skoog medium, including vitamins	Duchefa Biochemie, Haarlem	M0222.0025		25 L	Concentration: 4405.19 mg/L
MES (2-(*N*-morpholino) ethanesulfonic acid)	Duchefa Biochemie, Haarlem	M1503.1000	C6H13NO4S.H2O	1 L	
Daishin agar	Duchefa Biochemie, Haarlem	D1004.1000		1 L	
Sucrose	Duchefa Biochemie, Haarlem	S0809.5000	C12H22O11	5 L	
Bleach	Van Dam, Bodegraven	590	NaOCl	1 L	Brand: Piek / Concentration 4 g / 100 mL

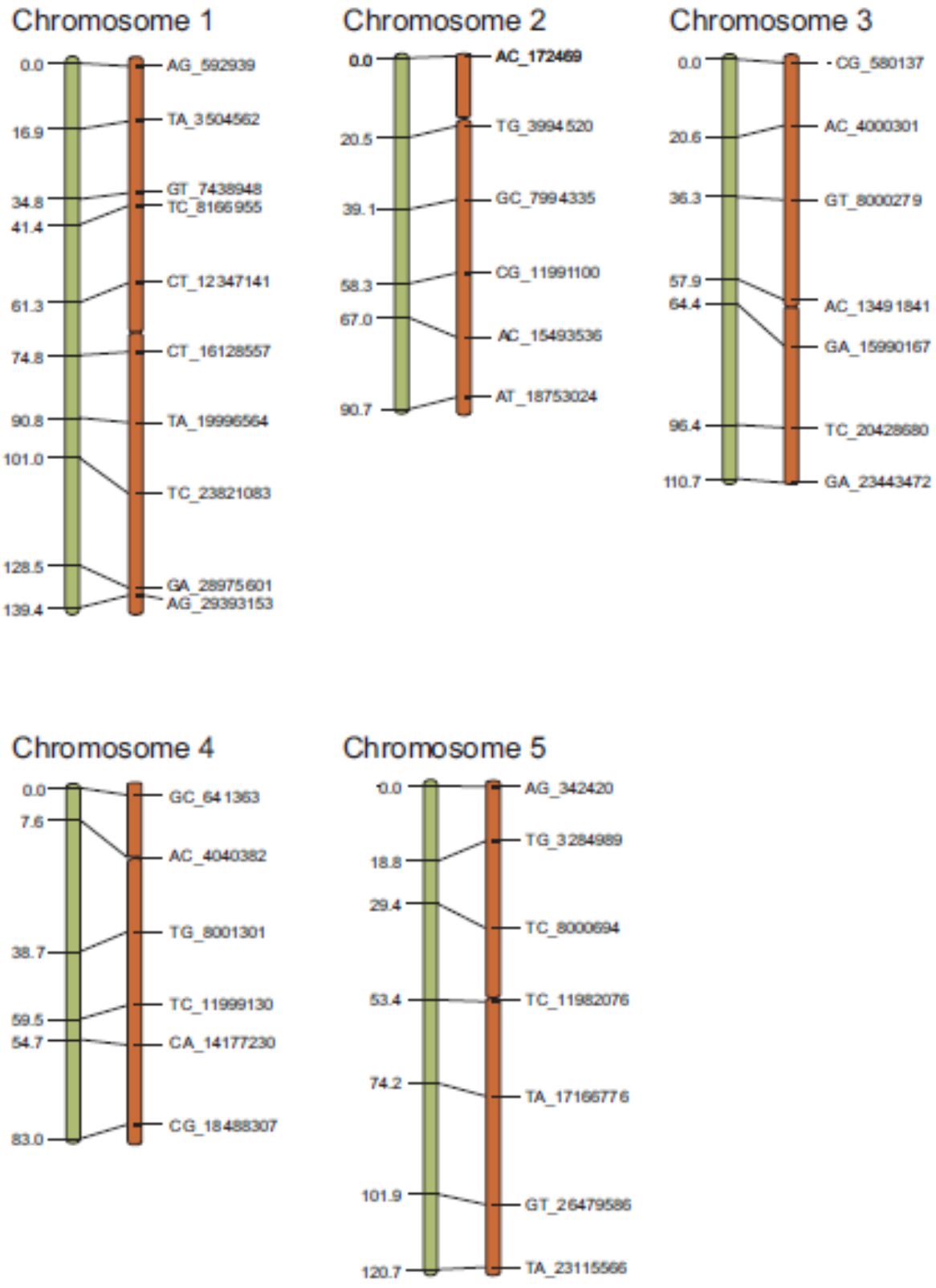

Figure 15 Genetic and physical map of *Arabidopsis thaliana* composed by Erik Wijnker (2010).

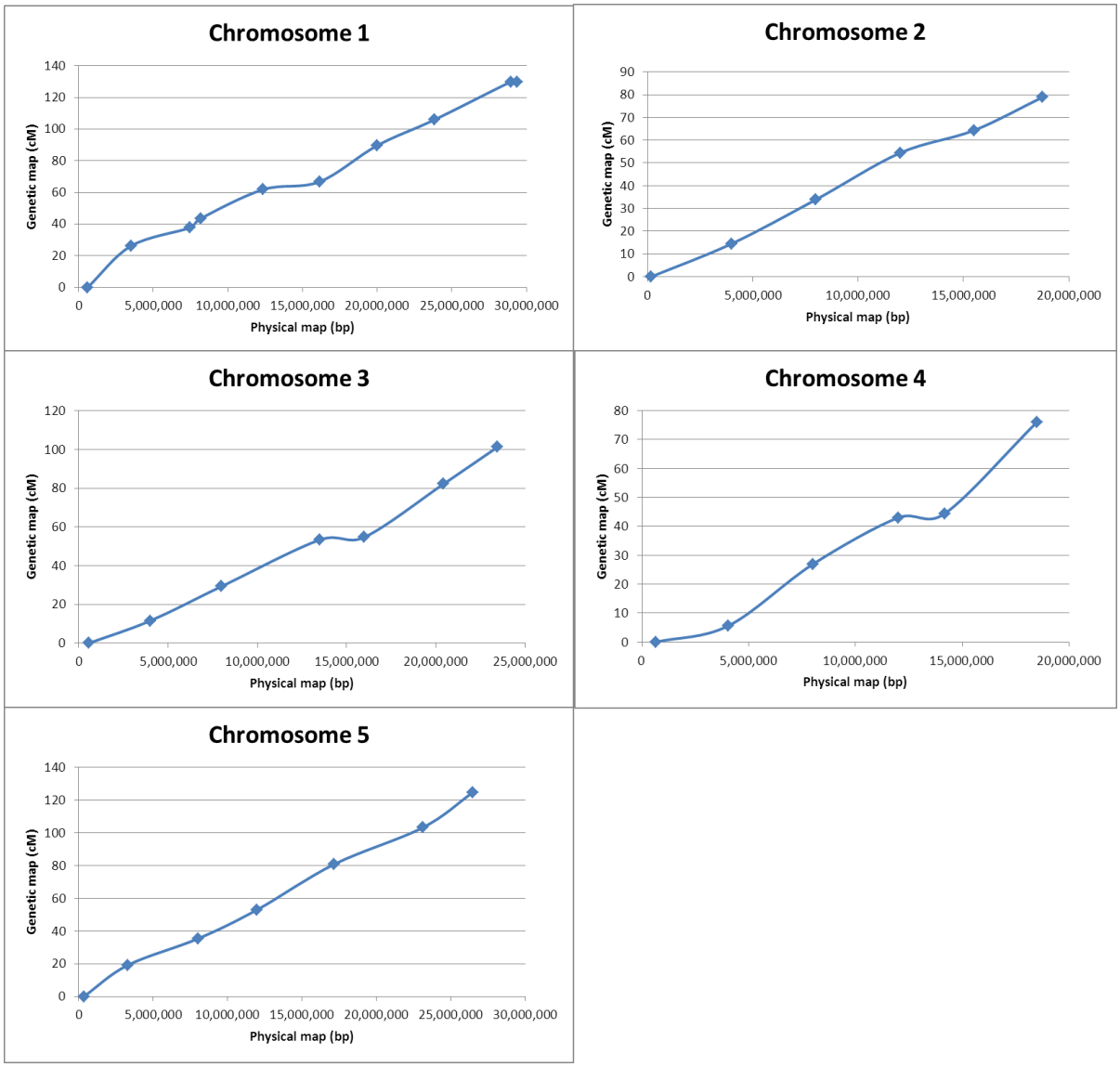

Figure 2 The genetic map versus the physical map, to observe the normalized crossover frequency. A flat line means, lower crossover frequency is present between these markers. A more steep line means higher crossover frequency occurs between the markers. Chromosome 2 and 5 is a very good example of a chromosome where all crossovers occur more or less on the same frequency. Chromosome 1, 3 and 4 shows all 1 position, where the line is flat.

	Chromosome 1										
Marker position	592.939	3.504.562	7.438.948	8.166.965	12.347.141	16.128.557	19.996.564	23.821.083	28.975.601	29.393.153	Total
Marker	B	H	A	A	A	A	A	H	H	H	
Lengthe between markers	592.939	2.911.623	3.934.386	728.017	4.180.176	3.781.416	3.868.007	3.824.519	5.154.518	417.552	1.034.518	30.427.671
Length recombination spot marker	2.048.751	3.423.005	2.331.202	2.454.097	3.980.796	3.824.712	3.846.263	4.489.519	2.786.035	1.243.294		30.427.671
Check												
Homozygosity	2.048.751		2.331.202	2.454.097	3.980.796	3.824.712	3.846.263					18.485.819 60,8%
Heterozygosity		3.423.005						4.489.519	2.786.035	1.243.294		11.941.852 39,2%
Total												30.427.672

Figure 1 Schematic overview of calculation of the amount of homozygosity of F2 plants, SDR plants and FDR plants. The marker position in base pairs is known and given in the figure. Therefore the amount of base pairs between the markers is calculated. If one markers scored homozygous and at least one of his neighbour scored heterozygous. The middle (in base pairs) between this markers is taken as standardize point to calculate the amount of homo- and heterozygosity. In the figure, the amount of homozygosity is calculated for a SDR-0 plant.

		Chromosome 1										Chromosome 2					Chromosome 3							Chromosome 4					Chromosome 5						
		592939	3504562	7438948	8166965	12347141	16128557	19996564	23821083	28975601	29393153	172469	3994520	7994335	11991100	15493536	18753024	580137	4000301	8000279	13491841	15990167	20428680	23443472	641363	4040382	8001301	11999130	14177230	18488307	342420	3284989	8000694	11982076	17166776	23115566	26479586
	Meiosis	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	38	D9	H7
Complementing SDR 1	76	H	A	A	A	A	A	H	H	H	H	A	A	H	H	H	H	H	A	A	A	A	A	H	A	A	H	H	H	B	H	B	B	B	H	H	H
Complemeniting SDR 2	65	H	B	B	B	B	B	H	H	H	H	H	B	B	B	B	B	H	B	B	B	B	H	H	B	B	B	B	B	H	A	A	A	A	H	H	H
Complementing F2 plant 1	14	B	B	B	B	A	A	A	H	H	A	H	B	H	A	A	A	H	H	H	B	B	B	B	H	H	H	H	H	B	A	A	H	H	H	B	B
Complementing F2 plant 2	99	H	H	H	A	A	H	H	H	H	H	H	H	B	B	B	B	H	H	A	H	H	H	H	H	A	H	H	H	H	B	B	B	B	H	A	A

Figure 2 Constructed complementing SDR-0 plants and F2 plants. The SDR-0 plants are derived from meiosis 76 and 65 of the cross between F1 hybrid (Col x L*er*) in the *quartet*-1 background with the male sterile Cvi-0. These SDR-0s are complementary for the centromeres (black markers). The F2 plants are selected for as much as possible complementarity. In the figure for one marker heterozygosity is lost (scored two times A). The F2 plants also show less fixation of heterozygosity. The amount of fixed heterozygosity of the complementing SDRs and F2 plants is calculated just like the above described method of calculating homo- and heterozygosity.

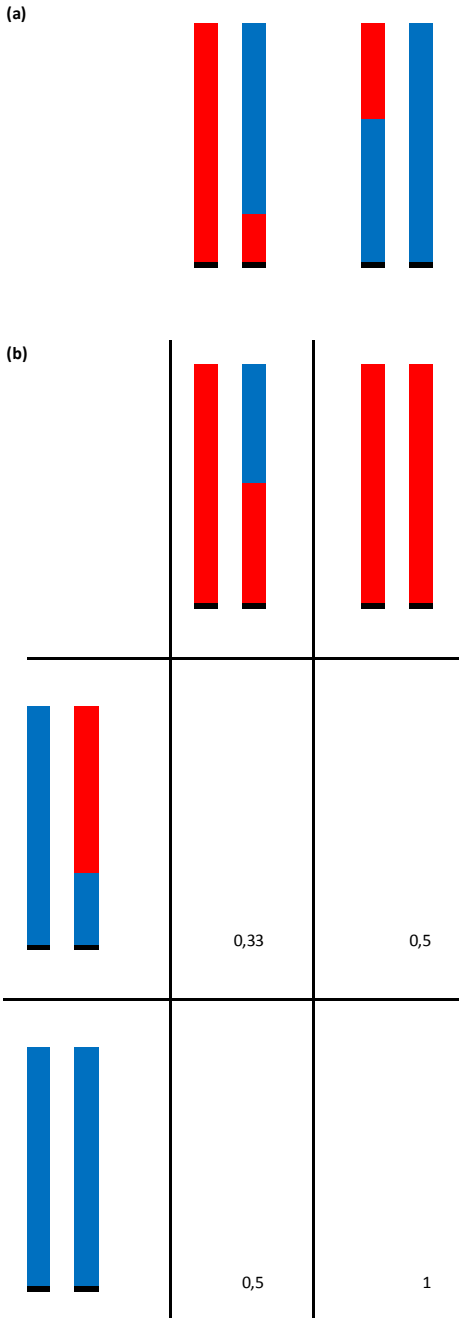

Figure 3 Schematical overview of the theoretical approach of fixation of heterozygosity in complementing SDR-0s. In (a), two possible complementing lines are depicted. Random crossovers with one crossover per chromosome arm occurred. The average amount of fixed heterozygosity in these plants is based on the SDR with the crossover the most close to the centromere. (In this case, the one which scored red for the centromere (centromere is black bar). The average amount of fixed heterozygosity in complementing SDR-0 plants is 33%. In (b) the average amount of fixed heterozygosity is shown for complementing SDR-0 plants with a crossover frequency of 1 crossover per 2 chromosome arms. In this case, different SDR-0 plants could be made. SDRs where the crossover occurs randomly over the chromosome and SDRs were no crossovers occurs. The table illustrate that with these possible SDRs, 4 different complementing combinations could be made. The average amount of fixed heterozygosity is also shown per combination. In total if one crossover per two chromosome arms occurs, amount of fixed heterozygosity is on average (100% + 50% + 50% + 33%) / 4 = 58%.