# Analysis of Chromosome Behavior in Four Achiasmatic *Arabidopsis thaliana* transformants for Reverse Breeding

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# Abbreviations

DH - double haploid

SC – synaptonemal complex

RNAi / siRNA – RNA interference / small interfering RNA

DSB – Double Strand Break

DN – Dominant Negative

DAPI - 4',6-diamidino-2-phenylindole

Col – Columbia (accession)

FISH – Fluorescence *in situ* hybridization

SSC – saline-sodium citrate buffer (3 M NaCl, 0.3 M sodium citrate, pH 7)

EDTA – ethylene diamine tetra acetic acid

TNB – Tris-NaCl Block (blocking buffer)

TNT – Tris-NaCl-Tween 20 (wash buffer; 0.1M Tris-HCl, 0.15M NaCl, 0.05% Tween 20, pH 7.5)

BAC – Bacteria Artificial Chromosome

# Abstract

Reverse Breeding offers a novel method for the regeneration of parental lines from selected heterozygous F1 hybrid. This is done by reducing the complexity of meiosis during meiosis by suppressing cross-over formation. *DMC1*, *SDS* and *ASY1* genes, that are responsible for synapsis and cross-over formation, are silenced by either RNAi or dominant negative (DN) construct. DAPI staining shows that these transformants behave similar to their respective mutant phenotype, with respect to showing asynapsis in prophase, no or low chiasma formation, and irregular chromosome segregation in meiosis I. These transformants also result in unbalanced gametes and reduced fertility. Chromosome painting using FISH techniques on RNAi-*DMC1*, DN-*DMC1* and RNAi-*SDS* reveals aberrations during first meiotic division that further elucidate chromosome behavior in achiasmatic meiosis. According to statistics, chromosome behavior at metaphase I is random, (10-0 to 5-5 segregation) but RNAi-*SDS* has a high frequency of cells with five chromosomes, tested by Chi-square analysis. Both *DMC1* transformants shows a high incidence of nucleus size 1, lagging chromosomes. FISH analysis suggested the behavior of lagging chromosomes in second meiotic division that result in unequal chromatid content in the tetrad stage for some transformants. Early chromatid segregation (ECS) is also observed mainly in *DMC1* transformants. Based on the analysis of metaphase II meiocytes of these transformants, RNAi-*SDS* has the highest amount of balanced dyad, 9% (4/44 cells). With respect to Reverse Breeding application, RNAi-*SDS* could be a promising candidate for cross-over formation suppression. This is due to having a high frequency of nuclei with five chromosomes after the first meiotic division, high percentage of balanced dyads and displaying fewer anomalies (no ECS observed) compared to the *DMC1* transformants. Further analysis such as finding the bivalent frequency could provide more information to make a corrected chromosome segregation analysis for these transformants. A tetrad stage FISH-analysis using BACs as probes would also give a more accurate analysis and amount of balanced gametes production at the end of meiosis.

# Chapter I: Introduction

Plant breeding aims at improving the quality and productivity of crops and to breed varieties that are more adaptable in new and changing environments. Before 1900s, plant breeding was based on “the breeder’s eyes”, selecting the best crops that are mostly high yielding and best in appearances to be sown in bulk and re-selected in the next generation. As plant breeding evolved with proper management schemes, specific crosses that combined new valuable traits in the next generation were recorded and assessed. Then, in the early 1900’s, a study on crosses between two pure inbred lines of maize introduced the hybrid breeding technique and this boosted agricultural production tremendously (Shull 1909, cited in Chahal and Gosal 2006 pp. 271-276). Hybrids are made from planned crosses of carefully chosen inbred parents, which are homozygous in almost all loci. This results in a heterozygous F1 hybrid variety that is more vigorous than their inbred parents, known as heterosis effect. A hybrid breeding program starts from the selection of inbred populations. The selected parental inbred populations are maintained through continuous inbreeding by self pollination. They are then tested for their combining ability to see which parents combination results in the best hybrid, based on a test-cross performance. The parental lines can be improved by introgressing traits from donor plants of the same species or even genus, followed by several generations of backcrossing to the parental line. The improved parental lines can be used again in the cross to produce new hybrid combinations.

In case superior hybrids or heterozygous plants are uncharacterized, for example in open pollinated population, it is difficult to reproduce these hybrids with existing plant

breeding techniques. The favorable allele combinations in these hybrids will be broken apart in the next generation due to segregation of traits in meiosis. There is an interest in plant breeding to come up with a technique that preserves the heterozygous genotypes into two homozygous plants that, upon crossing, will regenerate the same starting hybrid again (Wijnker and de Jong 2008). A technique called Reverse Breeding provides a method to enable the generation of parental lines for a selected heterozygous hybrid (Dirks et al 2003). Other than that, Reverse Breeding also offers a breeding approach referred to as breeding per chromosome which allows chromosome substitutions between two known homozygous plants in every possible way (Dirks et al, unpublished). By having chromosome substitution lines, and making crosses with them, breeders can control what chromosomes are present in homozygous or heterozygous states. Furthermore, breeders can produce populations that segregate for traits on only one chromosome through the breeding per chromosome technique. In short, Reverse Breeding allows breeders to have control over homo/heterozygosity per chromosome pair. Reverse Breeding begins with the suppression of cross-over formation in the selected hybrid. This is an essential step in Reverse Breeding, for it preserves the integrity of maternal and paternal homologs during meiosis. This is followed by the generation of double haploid (DH) from the spores containing non-recombinant chromosomes. Under the right *in vitro* conditions, viable spores will undergo spontaneous genome doubling to produce double haploids (homozygous diploid plants). Among these DHs, there are at least two DHs that contain the non-recombinant paternal and maternal chromosomes of the hybrid that, upon crossing, will reconstitute the same genotype as the starting hybrid.

Screening of complementing DHs is done by molecular genotyping. A figure explaining Reverse Breeding is depicted in Appendix 1.

Achiasmatic meiosis can be induced by silencing meiotic genes that are important in cross-over formation. One method is by using RNA interference (RNAi) or siRNA that result in post-transcriptional gene silencing. Studies on RNAi silencing of early meiotic genes such as *DMC1* can lead to suppression of crossover formation (Siaud *et al* 2004). Another option is using dominant-negative suppression: inserting a construct that encodes a truncated protein. Such a protein can disrupt the functioning of the endogenous proteins by preventing proper multimer formation. This results in a loss of function. In the human DMC1 protein, a deletion of 81 amino acids at the N-terminal results in the formation of heptamers (as opposed to octameres) and defects in dsDNA and ssDNA binding activities (Kinebuchi *et al* 2004). Other gene silencing techniques such as chemical silencing (Dupré *et al* 2008) and transmission of silencing constructs via grafting are also considerable alternatives to silencing gene in crops where the method of transformation is still unknown. The DH products from transgene-mediated techniques in Reverse Breeding can actually be transgene free as well. The silencing constructs are transferred in hemizygous state and thus, half of the spores will not contain the construct. Practically, the production of transgene-free DHs for complementing parental lines is relevant to the Reverse Breeding because they show normal meiosis and can be used in further improvements of parental lines.

As a result of suppressing cross-over formation, non recombinant parental chromatids are transmitted through each spore. In achiasmatic meiosis, all chromosomes have a chance of ½ to migrate to the same or opposite poles. Achiasmatic meiosis produces mostly

aneuploid, but among the euploids there is a probability of $(1/2)^x$ (x = basic chromosome number) for regular segregation of all chromosome pairs. In the case where silencing is not complete and there are residual cross-over formations, there will be some recombinant chromatids produced but these can be selected against using genetic markers. Provided that there is only one cross-over per chromosome, half of the spores still contain non-recombinant chromatids, which are still useful for Reverse Breeding. The chance for regular segregation will increase due to having residual cross-over formation.

Suppression of cross-over formation will result in production of unbalanced spores and consequently, reduced fertility (Wijnker and de Jong 2008). This highlights an important function of cross-overs for the production of balanced gametes.

The coordination of chromosome behavior depends on a series of complex processes which include homolog alignment, synaptonemal complex (SC) assembly, crossover recombination and chiasmata formation (physical connection at cross-over sites)(Roeder 1997). These processes occur during meiotic prophase I, which is divided into five substages; leptotene, zygotene, pachytene, diplotene and diakinesis. After these stages, homologs are held in pairs (bivalents) by the chiasmata until metaphase I, leading to equal segregation in anaphase I. The first meiotic division (reductional division) produces two daughter cells separated by a thin cell plate (rice), a phragmoplast membrane (tomato), or a cytoplasmic organelle band ( *Arabidopsis*) (Chen *et al* 2005; Havekes *et al* 1994; Ross *et al* 1996). Until metaphase II, the sister chromatids are bound by cohesion proteins (cohesins) at the centromeres (Ma 2006). At anaphase II, sister chromatids separate as the cohesins break down (Ross *et al* 1996) and move to opposite poles in the

second meiotic division, producing four haploid nuclei: the tetrads. Finally, the miscrospores are divided by cytokinesis and develop into mature pollen grains.

The correct course of meiosis depends on some important features. Firstly, during prophase I, homologous chromosomes are kept together by a proteinaceaous structure called the synaptonemal complex (Pradillo *et al* 2007). The mechanism of pairing varies depending on the organism; some have the full alignments of homologs of the whole chromosome before synapsis, some undergo sequential homologous alignment and synapsis segment by segment (Roeder 1997). In *Arabidopsis*, full homologous alignment is needed before synapsis. The axial elements (AE, one of the components of the synaptonemal complex), begin to develop as early as in the leptotene stage. Then, homologs align in zygotene "presynaptic alignment" from the terminal regions to the centromere. (Lopez *et al* 2008). Homolog recognition and alignment of AE's (later named lateral elements (LE)) lead to synapsis through tranverse filament (components of SC) polymerization (review Mercier and Grelon 2008). Some of the proteins that constitute the most of tranverse filament elements are *ZYP1* as the central element (Osman *et al* 2006) and *ASY1* as the axial-associated proteins (Armstrong *et al* 2002). Study of RNAi silencing of *ZYP1* in *Arabidopsis* resulted in 20% reduction in cross-over formation and high levels of non-homologous pairing (Higgins *et al* 2005). In pollen mother cells of the *asy1* mutant of *Arabidopsis*, synapsis is reduced, resulting in low number of bivalents (1.57 bivalent per cell), and a high proportion of unbalanced gametes and sterility (Ross *et al* 1997).

As in most organisms, meiotic recombination and synapsis in plants are tightly linked processes. Study of the ZYP1 protein in *Arabidopsis* reveals that the association of SC

components during synapsis depends on double strand break (DSB) formation, which is triggered by other proteins, AtSPO11-1 and AtPRD1(Higgins *et al* 2005, De Muyt *et al* 2007). Mutant studies of these genes show an inability to form cross-overs, leading to asynaptic chromosomes, univalents and unbalanced segregation. Another gene that has a similar function based on the mutant phenotype is a cyclin-like protein, SDS (Azumi *et al* 2002). DSB formation is a conserved process among eukaryotes required for the initiation of meiotic recombination. The DSBs induced by endonuclease proteins such as *SPO11-1,* are first resected to produce single-strand ends. These single-strand ends are used by recombinase enzymes, such as *DMC1*, to invade the double helix of non-sister chromatids at homologous sites (Tsubouchi and Roeder 2003). DMC1 functions specifically in catalyzing strand exchange and DSB repair between homologous chromosomes during meiotic recombination (Klimyuk and Jones 1997). Defects in *DMC1* result in reduced meiotic recombination, formation of univalents and unbalanced chromosome segregation (Couteau *et al* 1999).

The DSB repair mechanism produces a molecular structure known as the Holliday junction. This structure resolves in two ways, based on a Double Strand Break (DSB) model; a vertical resolution of the Holliday junction will produce a cross-over whereas a horizontal resolution leads to gene conversion (Appendix 2). Crossing-over will result in reciprocal recombination between homologous chromosomes, which is crucial for the formation of chiasmata (physical connections between homologs) that are visible in late prophase I (diplotene-diakinesis). In mutants where cross-over and recombination are disrupted, it is also observed that chromosomes have no or reduced chiasmata (review Mercier and Grelon 2008). Absence of chiasma in plants leads to unbalanced

chromosome segregation. Different from what is observed in plants, balanced segregation of achiasmate chromosomes is observed in chromosome 4 of *Drosophila* (Hawley *et al* 1993).

Reverse breeding is based on producing balanced gametes containing non-recombinant chromosomes. The feasibility of inducing asynapsis and suppression of cross-over formation is the focus of this thesis. Therefore we will analyze transformants in which the genes *DMC1*, *SDS* and *ASY1* were silenced. These genes were selected for showing univalent phenotypes (review Mercier and Grelon 2008). We will describe the meiotic progression of these four *Arabidopsis* transformants (RNAi-*DMC1*, Dominant Negative-*DMC1*, RNAi-*SDS* and RNAi-*ASY1*) by construction of meiotic maps of pollen mother cells from early prophase I to the tetrad stage (Results section II). Futher descriptive analyses on chromosome behavior of these achiasmatic transformants will be done by chromosome painting techniques (Results section III). Using fluorescent probes we identify the homolog pairs in *Arabidopsis*, enabling a detailed study of chromosome segregation during meiosis I. To achieve this, we will first discuss and evaluate various painting schemes based on experimental results (Results section I).

This thesis aims to answer the following questions:

1) Does the silencing of *DMC1*, *SDS* and *ASY1* work in the four transformants? In other words, are we able to induce a phenotype in these plants that concurs with the description of their respective mutants?
2) Is univalent segregation random (as expected) or non- random? Chromosome painting schemes will be applied followed by statistical analysis and graphical

representations will be presented to visualize chromosome segregation pattern after the first meiotic division.

3) What gene should be silenced for Reverse Breeding? In other words, which silencing construct works best? Based on the analyses, we are interested in finding which transformant/s produce the high percentage of balance gametes, containing non-recombinant chromosomes. Besides that, we are interested in avoiding additional meiotic irregularities, We want to induce random chromosome assortment without damaging the chromosome structure, and having lagging chromosomes and early chromatid segregation events.

# Chapter 2: Materials and Methods

## *I. Plant Material*

Seedlings of wild type *Arabidopsis thaliana* (Col) and transformants (Col) containing transgenes (Table 2.1) were provided by Rijk Zwaan Co.

**Table 2.1**: List of transformants with their respective silencing construct

| **Transformant** | **Silencing construct** |
|---|---|
| pRZ051 | RNAi-*DMC1* |
| pRZ256 | Dominant-negative *DMC1* |
| pRZ150 | RNAi-*SDS* |
| pRZ151 | RNAi-*ASY1* |

The DN-*DMC1* construct produces *DMC1* protein lacking in N-terminal domain, which is important in the octamer formation of the protein and helps in DNA binding (Kinebuchi *et al* 2005). The constructs also contain a kanamycin resistant gene for selection. For details of transformation see the patent text of Reverse Breeding (Dirks et al 2003 pp. 13-16).

Both RNAi-*DMC1* and dominant-negative *DMC1* seedlings populations were obtained by selfing the primary transformants. The seeds were vernalized in ½ MS medium containing kanamycin in $4^{o}$C and germinated in climate chamber. Then, the seedlings were selected for kanamycin resistance indicating they contain the T-DNA. The resistant seedlings populations showing a 6-3:1 (resistant:not resistant) segregation pattern were selected and transferred to the greenhouse until flowering. RNAi-*SDS* and RNAi-*ASY1* were primary transformant ($T_0$) seedlings; therefore we did not test for segregation of resistant marker. We selected the seedlings that showed the most sterile phenotype (short siliques, low seed count) to indicate that the T-DNA construct is present and well expressed.

## *II. Slide preparation*

*Fixation*: After growing for 4-5 weeks, flower buds were collected from phenotypically sterile plants, recognizable by having small siliques. Five to eight inflorescences for eight randomly selected sterile plants were obtained. WT flower buds (Col) were collected and fixed for comparison. For each transformant, the list of selected plants is presented in Table 2.2.

**Table 2.2**: List of selected plants for each transformant

| RNAi-*DMC1* | DN-*DMC1* | RNAi-*SDS* | RNAi-*ASY1* |
|---|---|---|---|
| 051-34-32 | 256-32-8 | 150-1 | 151-10 |
| 051-34-1 | 256-32-14 | 150-8 | 151-11 |
| 015-39-3 | 256-35-3 | 150-12 | 151-13 |
| 051-39-39 | 256-36-21 | 150-19 | 151-22 |
| 051-47-31 | 256-36-32 | 150-22 | 151-26 |
| 051-48-33 | 256-38-23 | 150-30 | 151-27 |
| 051-62-29 | 256-47-19 | 150-32 | 151-36 |
| 051-62-45 | 256-47-25 | 150-36 | 151-40. |

Flower buds were put in Carnoy's fixative (absolute ethanol:glacial acetic acid: 3:1) overnight at 4$^{o}$C. The fixative was then changed to 70% ethanol and kept in 4$^{o}$C until required.

*Cell spreading*: Fixed buds were washed twice with Milli-Q (5 minutes each), followed by two times 5 minutes washing with 10mM citrate buffer (pH 4.5). Flowers with yellowish anthers were discarded because those contain mature pollen. Buds in stages 9-11 of floral development according to Smyth *et al* (1990) were chosen for meiosis II analysis in pollen mother cells. Buds were incubated in a small petri dish with 10x dilution of a 1% PCC enzyme mixture (0.3% w/v pectolyase, 0.3% w/v cytohelicase and 0.3% w/v cellulase) for 3.5 hours at 37$^{o}$C. The digestion was checked by tapping one flower bud on a slide and if the anthers fell out easily, digestion was completed. The enzyme mixture was replaced with cold citrate buffer and the small petri dish containing

buds was put on ice to halt the reaction. A single bud was transferred to a clean, dry glass slide (kept in 70% ethanol) and 1-2µl Milli-Q water was added to keep the flower moist. The bud was tapped gently with a rod to release pollen mother cells from the anthers until the cell solution was homogenized. Sometimes small amounts of Milli-Q water was were added in a small amount if the to prevent dehydration of the cell suspension. One or two drops of 50% acetic acid were added to the cell suspension. Cells were spread by gently moving a pointed metal rod placed parallel to the slide surface on 45$^{o}$C hot plate for 1 minute, trying to promote precipitation of cells in a small area, small enough to be covered with the cover slip afterwards. Cells were refixed with fresh ice-cold 3:1 Carnoy's solution (ethanol: acetic acid) by placing drops of Carnoy's solution around the area where cells were spread to ensure that the cells precipitated in the intended area instead of becoming fixed at the slided edge. Preparations were kept in dark boxes in 4$^{o}$C until needed for chromosome painting.

*DAPI staining and microscopy*: Selected slides were stained by adding 12µl 1:20 DAPI in Vectashield mounting medium and covered with 24x32 mm cover slip. DAPI stained slides were observed and photographed using Nikon (*model)* camera and processed using the program Image Pro Plus program. Images were improved with the Photoshop CS3 software (Adobe Systems, San Jose, CA).

## *III. Fluorescence in-situ hybridization (FISH)*

*Selecting slides*: Slides with mostly dyad or tetrad stages were identified and selected by phase contrast microscope. It is critical to be able to distinguish meiocytes from other cells that are abundant on the preparation. By using 40 X or 63 X magnifications in a phase contrast microscope, condensed chromosomes (in diakinesis, metaphase, and anaphase) looked like black, elliptic spots.

*Quality assessment of DNA or probes for FISH*

*a) DNA purity*

All DNA in this experiment were isolated using the Roche Plasmid Isolation Kit following the manufacturer's protocol with minor modification in the elution step. Instead of adding 100µl of elution buffer, 30µl Milli-Q water was added to each collection tube to elute the DNA.

A DNA purity check was done after the isolation procedure by pipetting 1:1 Loading Dye and DNA in each well (1 µl = 30-100ng DNA). Usually, when DNA was isolated using the kit, the yield was clean, showing a clear band.

*b) DNA concentration*

Checking post-DNA isolation yield on the gel electrophoresis does not give a definite measurement of how much DNA obtained from an isolation event. Usually 50ng -1 µg of DNA is needed for hybridization in FISH experiment. DNA concentration can be measured using a NanoDrop $^{TM}$ machine.

When DNA is isolated using the Roche kit, the usual yield would be around 15-30ng/µl for BACs and more than 25 ng/µl for plasmids containing rDNA (5S, 45S). To obtain a

sufficient amount of DNA, two to three isolation events were performed for each BAC and the yield was combined in one Eppendorf tube. Then, the solution was precipitated with a 2.5 volume absolute ethanol and 0.1 volume 3M sodium acetate pH 5.2. The mixture was vortexed well before putting at -20$^{o}$C overnight. After that, the mixture was spun down at 12000 rpm at 4$^{o}$C for 30 minutes. The supernatant was removed using a pipette and the pellet was air-dried at 37$^{o}$C. The pellet, usually invisible, was dissolved in 35µl of sterile Milli-Q water.

*c) DNA fragment size*

Checking the DNA on gel electrophoresis requires the knowledge of the expected fragment size to ensure we obtained the right isolation products. Also, after a Nick translation reaction it is wise to check whether the right fragment size was obtained.

Figure 2.1 shows what DNA bands look like on a gel when compared to a DNA ladder.

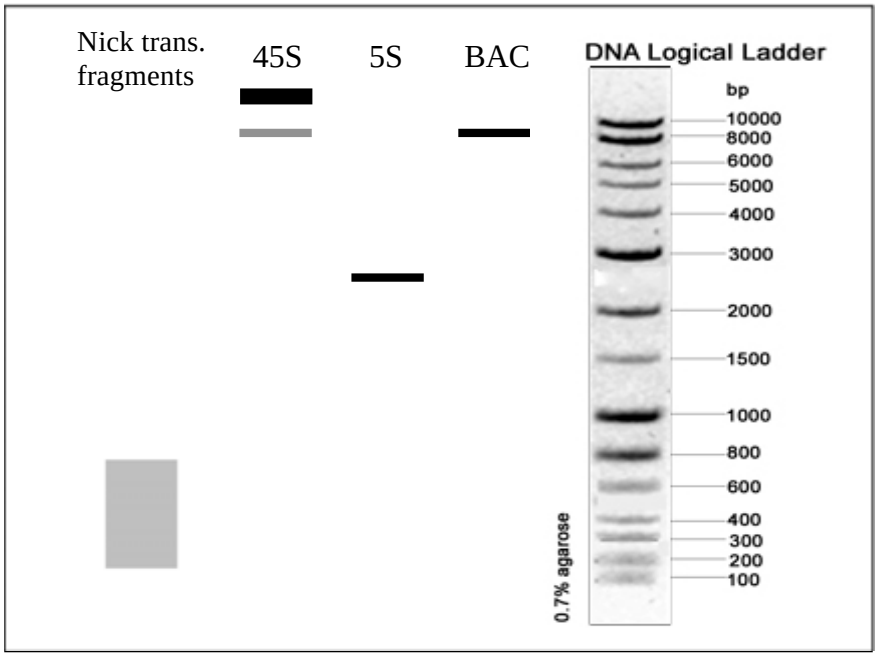



**Figure 2.1:** Comparison of DNA fragment sizes to the DNA Logical Ladder to check DNA and probe quality

*Nick Translation Reaction*

Isolated DNA was labeled by incorporating directly labeled dUTP/dNTP or digoxigenin/biotin dUTP in a nick translation reaction. Table 2.3 summarizes the amount of chemicals and DNA to be put inside one tube. Depending on the DNA isolation concentration, one reaction tube should at least contain 360ng (DNA for direct labeling and 480 ng DNA for indirect labeling. According to the protocol, there should be 1000 ng DNA per labelling reaction, but for a FISH experiment, a range of 50-1000 ng of DNA per slide is acceptable. The DNA probe from the reaction mix could be used for at least four slides (90ng/probe/slide). All chemicals were combined on ice. Sterile Milli Q water was used to make up the final volume in each tube to be 20µl. After adding all the chemicals accordingly, the mixture was incubated at 15$^{o}$C for 1.5 hours. After 75 minutes, the reaction was checked whether it is completed by putting 1µl of nicked DNA plus 1µl Loading Dye in 1% Agarose gel (1x TAE, 1µl GelRed). A smear between 500bp – 200bp indicated the reaction was sufficient. If the bands produced were still larger than 1kb, then the reaction should continue on. The reaction was stopped by adding 1µl 0.5M EDTA, pH 8.0 followed by heating at 67$^{o}$C for 10 minutes. Tubes were wrapped in foil to shelter them from direct light.

**Table 2.3**: Chemicals combination in one eppendorf tube for DNA labeling via Nick Translation reaction.

| | Indirect DIG (later FITC) | Indirect Biotin (later Cy5) | Cy3.5 | Cy3 | DEAC |
|---|---|---|---|---|---|
| 300-500ng DNA + sterile MQ if necessary | 16µl | 16µl | 12µl | 12µl | 12µl |
| Nick Translation Mix (NTM) | 4µl DIG NTM | 4µl Bio NTM | 4µl NTM | 4µl NTM | 4µl NTM |
| dNTP Mix 1 (100mM) | - | - | - | 2µl | 2µl |
| dNTP Mix 2 (100mM) | - | - | 2µl | - | - |
| Labeled dNTP | - | - | 2µl | 2µl | 2µl |

*FISH experiment protocol*

After labeling the probe DNA, a hybridization mix was prepared by adding 4µl of every probe in one tube. The mixture was precipitated following the procedure described under "Quality assessment of DNA for FISH". After the pellet was dried, the pellet was resuspended in 10µl of HB50 and stored in -20$^{o}$C until required. Hybridization mix for different slides was prepared in separate tube.

Selected slides were heated at 65$^{o}$C for 30 minutes or at 37$^{o}$C overnight. Then, the slide was incubated at 37$^{o}$C for 1 hour with RNase (10mg/ml) diluted in 2X SSC (1:100) using a 24x50 cover slip. After the treatment, the slides were washed with 2X SSC three times, five minutes each wash. 1% formaldehyde buffer (50ml) was freshly prepared from 5ml 10X PBS, 5ml 10x $MgCl_2$ (500mM), 1.5ml 37% formaldehyde and 37.5ml Milli-Q. Slides were fixed in this buffer for 10 minutes in the fume hood, and washed with 2X SSC thrice, 5 minutes each wash. Next, slides were dried with ethanol series 70%-90%-100% for 3 minutes each. Meanwhile, 10µl of 20% Dextran sulfate was added to the probes mixture in HB50. Dextran sulfate is a viscous solution, so pipetting was done carefully. The mixture was vortexed well to avoid coagulations of probes. Hybridization mix was boiled in 100$^{o}$C water for 10 minutes followed by instant cooling in an ice bath. The 20µl of hybridization mix was pipetted onto the slide and covered with a 24X32 cover slip. Slides were denatured at 80$^{o}$C on a hot plate for 2 minutes, followed by over night incubation at 37$^{o}$C in a humid chamber. Next day, cover slides were removed with 2X SSC. In the meantime, the 50% formamide solution was warmed up to 42$^{o}$C. The slides were immersed in 50% formamide for 5 minutes three times. This was done in the

fume hood with extra care. Next, the slides were washed with 2X SSC for 5 minutes followed by washing with 4T (4X SSC +0.5% Tween 20) for 5 minutes.

For indirect labeled probes, the following signal amplification steps were required: 100 µl of detection solution mixing Cy5 in TNB blocking solution in 1:200µl ratio was put on the slide, covered with a 24X50 coverslip and incubated at 37$^{o}$C for 1 hour. Slides were washed with 4T once and twice in TNT (0.5% Tween 20 in 1X TN), five minutes each wash. The second incubation was with 1:25 µl biotinylated strept-avidin in TNB blocking solution and 1:200 µl sheep anti-dig-FITC in TNB for 1 hour at 37$^{o}$C. Slides were washed with TNT three times, five minutes each wash. The third incubation was with 1:200 µl Cy5 in TNB and 1:800 µl anti-sheep-FITC in TNB. After one hour, slides were washed with 2X SSC twice followed by drying with series of ethanol (70%-90%-100%) three minutes for each immersion. Preparations were counterstained with 1:20 DAPI in Vectashield antifade mounting medium using 24x32 coverslip and excess counterstain was removed by squeezing slides between several sheets of filter paper. Each slide was examined using epifluorescence microscopy, capture with Nikon (model) camera and images were processed using Image Pro Plus or Cytovision 3.6 program. Data of cells in dyad and tetrad stages were analyzed using in Microsoft Excel. Images were improved using Photoshop CS3 software (Adobe Systems, San Jose, CA).

# Chapter 3: Results

## *I. Chromosome painting scheme*

Two chromosome painting schemes were proposed to distinguish all five chromosomes of *Arabidopsis thaliana* in order to visualize homologous chromosomes in the products of the first and second meiotic division (dyad and tetrad stages) and explain the pattern and behavior of chromosomes during segregation. In a previous study, Pradillo *et al* (2007) showed that it is possible to identify three chromosome pairs in Arabidopsis by chromosome paints using rDNA.

In the first chromosome painting scheme, groups of BACs were selected for each chromosome and each group was labeled with a unique fluorophore. The labeling scheme, including the BAC numbers, is presented in Figure 3.1, with the names of the BACs per chromosome used in the FISH experiment.

BACs containing *Arabidopsis* inserts were chosen from our library based on their physical location on each chromosome (Appendix 3). These BACs are located far from the centromere region to avoid possible repeat rich region, and were chosen close to each other (at most 800kb apart) in order to obtain a clear signal for each chromosome after FISH. For chromosome 1, 4 and 5, direct labeling were used. Indirect labeling was applied to groups of two BACs located on chromosomes 2 and 3.

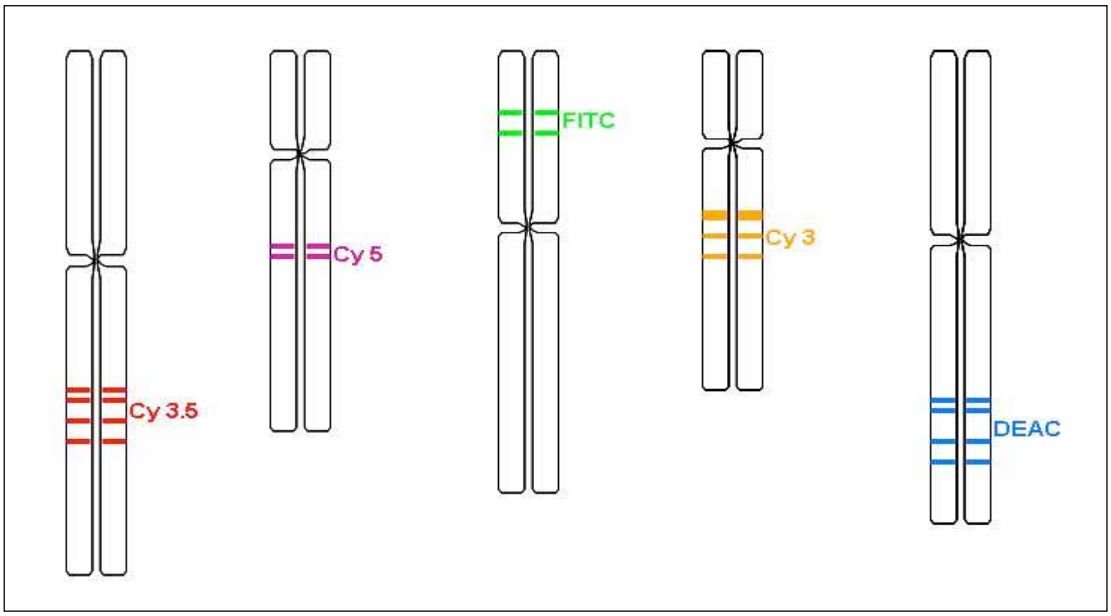



**Figure 3.1:** Chromosome painting scheme 1: From left to right- Chromosome 1 [T6H22, F13N6, T18I24, T30E16], Chromosome 2 [F23N11, F7O24], Chromosome 3 [MWL2, MJL14], Chromosome 4 [F8L21, F25E4, T20K18, F18A5], Chromosome 5 [K9B18, MSL3, K9H21, MVP7].

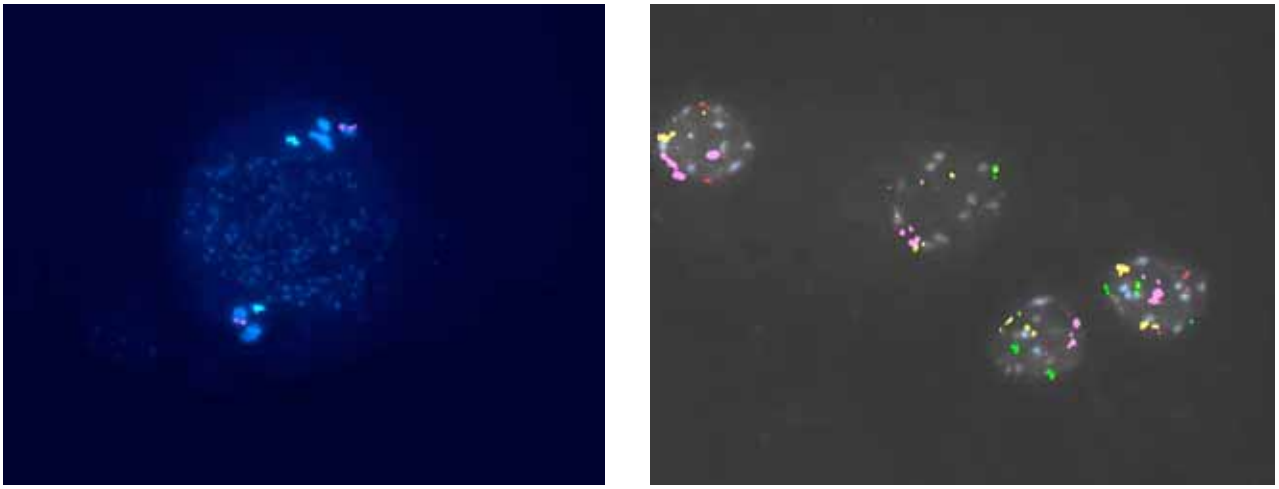


**Figure 3.2**: Result of chromosome painting labeling scheme 1 (Figure 3.1). **a** Metaphase II meiocyte with only two of the chromosomes labeled, in this case, chromosomes 2 and 3. **b** Interphase cells with all signal present (right most cell and upper left cell)

Probes did not hybridize on all chromosomes, allegedly due to poor quality of the DNA probes (Figure 3.2a). In most metaphase I cells, BAC's hybridized to only two or three chromosomes, and best results were obtained using indirect labelling technique. Labeling scheme 1 worked on meiocytes in meiotic phases with condensed chromosomes. The DEAC signal seemed to be present in all chromosomes especially in the pericentromeric

regions. This might be due to the leaking of DAPI signal to the DEAC channel (crosstalk) and this interfere with the interpretation of the DEAC signal. Therefore, DEAC probes should be far from the centromeres. The BACs used in this painting scheme were not contiguous (Appendix 3). Therefore, in cells with diffuse chromosomes, for example the tetrad stage, the different BACs appear as seperate spots (Figure 3.2a). This leads to imprecise interpretation, since it cannot be determined what signals belong to what chromosome.

The second painting scheme relied on rDNA loci. Using the rDNA probes, one can label four out of five *Arabidopsis* chromosomes. This chromosome painting scheme is only applicable to certain accessions like Col, Ler and Hey-0 because the 5S locus is absent in chromosome 3 in other accessions like Cvi and Ws (Sanchez-Moran *et al.* 2002).

We added two BACs on chromosome 1 and 3 because chromosome 1 does not have an rDNA loci, and to differentiate chromosome 3 from chromosome 5 (because both have 5S locus) (Figure 3.3).

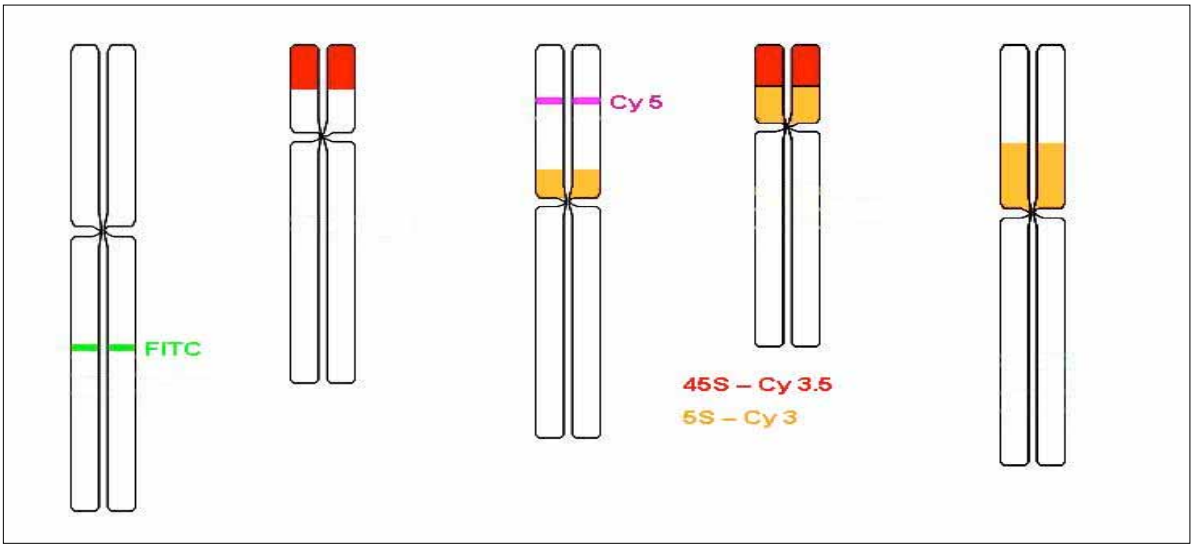



**Figure 3.3**: Chromosome painting scheme 2: From left to right – Chromosome 1 [F13N6], Chromosome 2 [pTa71 (45S rDNA)], Chromosome 3 [MWL2, pCT4.2 (5S rDNA)], Chromosome 4 [pTa71, pCT4.2 (45S and 5S rDNA)], Chromosome 5 [pCT4.2 (5S rDNA)].

Using the painting scheme proposed in Figure 3.3, all five chromosomes could be distinguished (Figure 3.4). In both condensed and diffused state (Figure 3.4a & b) the signals were distinctive. This scheme was employed for FISH analysis in three transformants (RNAi-*DMC1*, Dominant Negative – *DMC1* and RNAi-*SDS*) to visualize anomalies in achiasmatic meiosis and to analyze chromosome segregation.

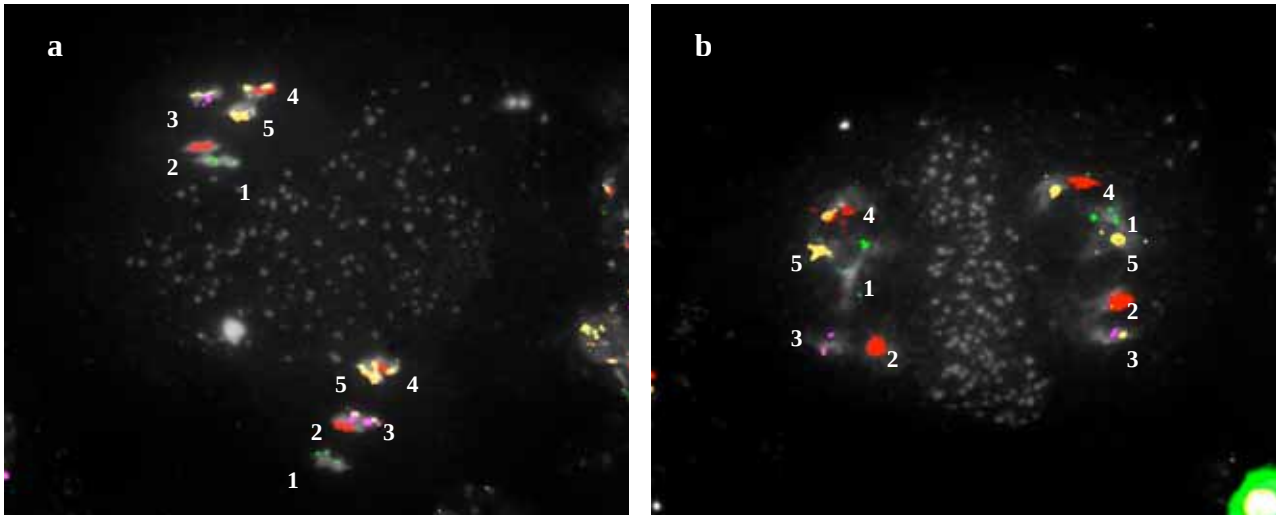
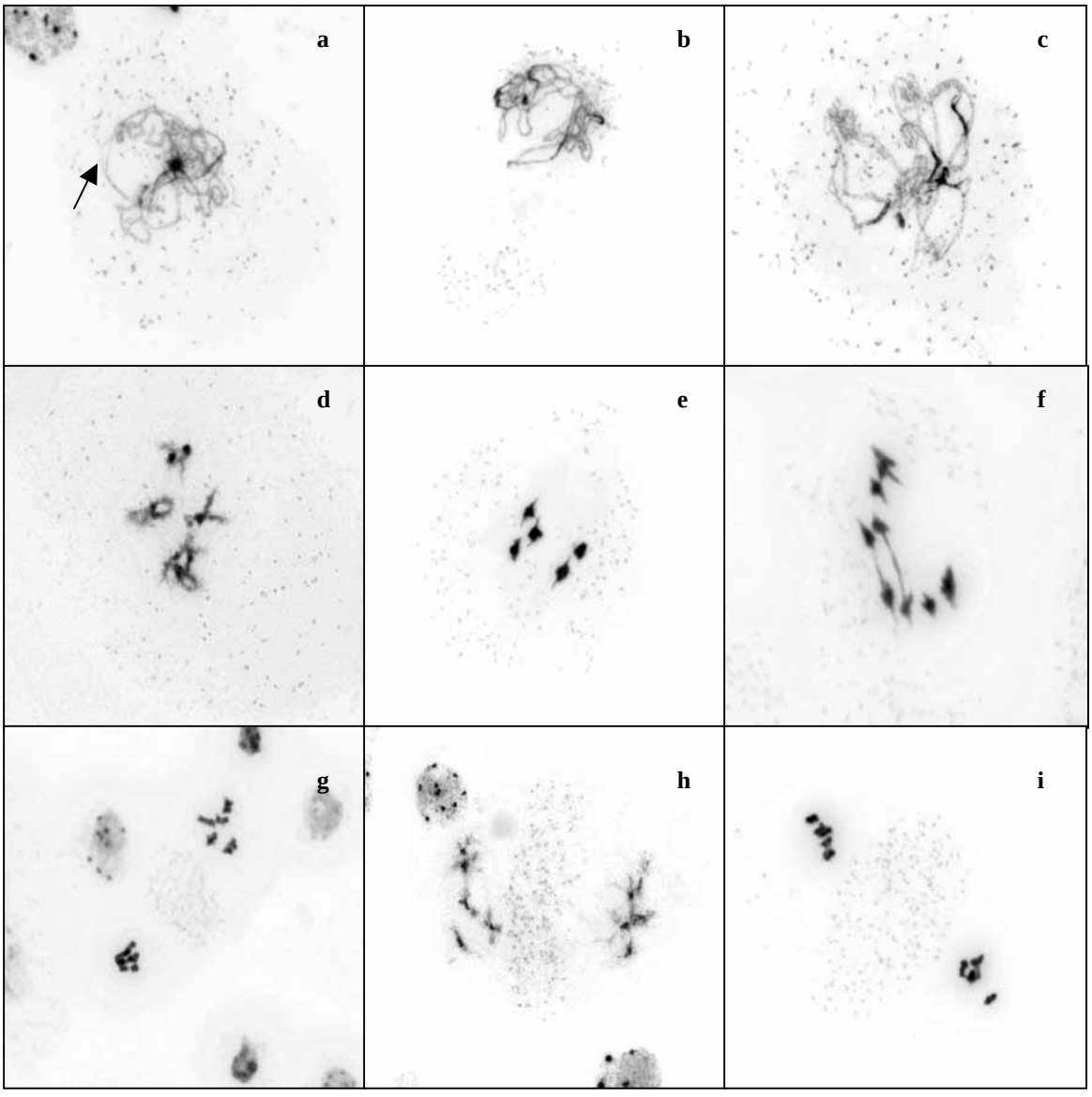



**Figure 3.4**: Result of chromosome painting using scheme 2: **a** Metaphase II cell with all five chromosomes labeled according to scheme 2. **b** Meiocyte in interkinesis stage, also showing all five chromosomes labeled.

## *II. Meiotic map of wild type Arabidopsis thaliana (Col) and four achiasmatic transformants*

Meiotic atlases were constructed from the images captured from DAPI stained preparations. A wild-type *Arabidopsis* meiosis atlas was fully described stage by stage in Ross *et al* (1996). Figure 3.5 shows the normal meiosis course starting from early prophase I until tetrad stage.



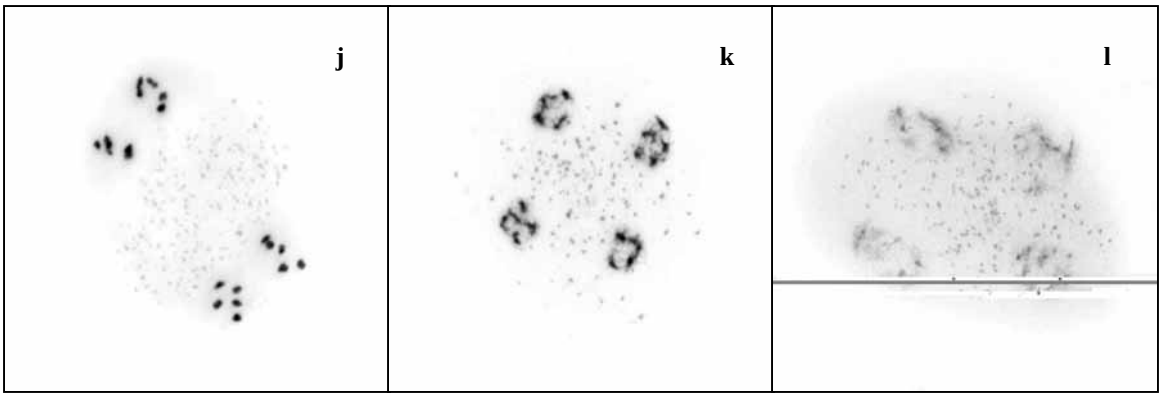



**Figure 3.5**: Meiotic stages from pollen mother cell spread followed by DAPI staining of *Arabidopsis thaliana*. **a** Zygotene showing synapsis in progress. Synizetic knot is prominent from which euchromatic DNA loops out. Arrow points to unsynapsed region. **b** Pachytene stage showing complete synapsis and clear five centromere (dark) regions. **c** Late pachytene, where homologous chromosomes are desynapsing. **d** Diakinesis stage with five bivalents, most showing two chiasmata. **e** Metaphase I with five condensed bivalents on equator. **f** Homologs segregate to opposite poles at anaphase I. Arrow points to string between two homologs indicating sister chromatid separation requires force. **g** Segregation finished at telophase I forming dyad with organelle band in the middle. **h-i** meiosis II begins with prophase II (h) and two groups of five chromosomes align at equator at metaphase II (j). **j** Anaphase II showing equational segregation to four groups. **k** Telophase II showing four nuclei. **l** tetrad stage.

In the zygotene stage (Figure 3.5a) the chromosomes are extended and grouped together in synizetic knot. At this stage, chromosome pairing and recombination usually takes place, notable by having synapsis due to SC assembly along the chromosome. Synapsed and unsynapsed regions are distinguishable as thick and thin threads. At the pachytene stage, homologous chromosomes become fully synapsed (Figure 3.5b). All five pericentromeric regions are heavily stained by DAPI. Bivalents become extended in early diplotene stage as the homologs desynapse and separate (Figure 3.5c). At diakinesis, homologs are fully separated and only attached by chiasma, the remnants of cross-over events (Figure 3.5d). The bivalents condense maximally at metaphase I and they are oriented in the equatorial plane with centromeres facing opposite poles (Figure 3.5e), ready to undergo the first meiotic division. In anaphase I, homologs consisting sister chromatids, migrate to opposite poles, reducing the chromosome number of the daughter cells (the reductional division). The segregating homologs show a link as a result of a

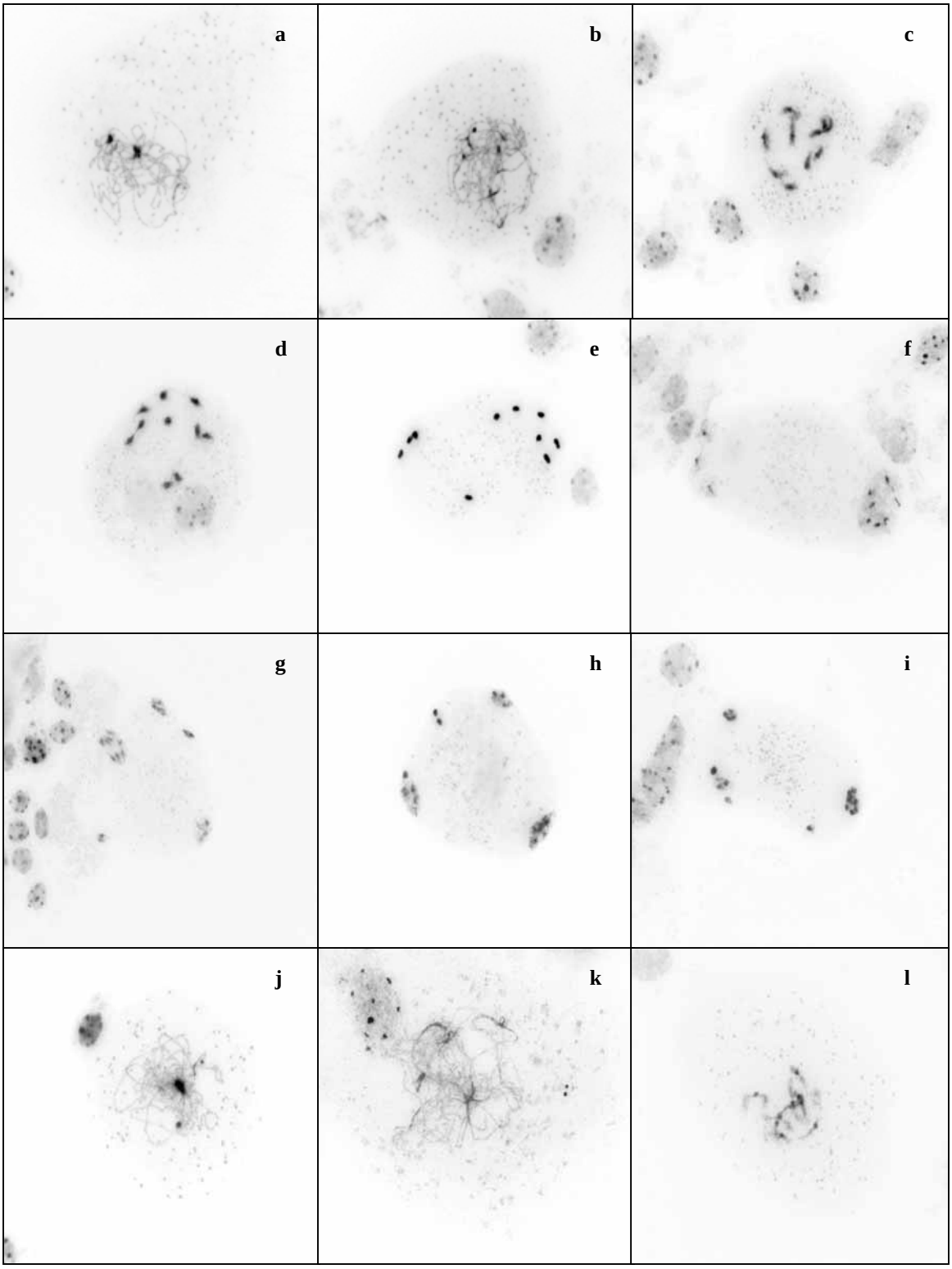
pulling force between them that indicates chromatid exchange between homologs before segregation (Figure 3.5f). After the first meiotic division, two nuclei containing five chromosomes are produced. These are separated by a cytoplasmic organelle band (Figure 3.5g). The second meiotic division starts after interkinesis (Figure 3.5h) and the chromosomes in each nucleus condense and align along the equator (Figure 3.5i). At anaphase II, homologs undergo equational segregation, resulting in the segregation of sister chromatids to opposite poles (Figure 3.5j) forming four groups of five chromatids (Figure 3.5k). The tetrad nuclei (Figure 3.5l) will be separated from one another by cytokinesis and will develop into pollen grains.

From the wild type meiotic map presented, it is clear that crossover formation joins homologs together and that ensures proper chromosome segregation throughout meiosis. Next, we present meiotic maps for *Arabidopsis* transformants that contain constructs to silence meiotic crossover formation. We describe these meiotic transformants: RNAi-*DMC1*, DN-*DMC1*, RNAi-*SDS* and RNAi-*ASY1*, and compared them to their respective mutant phenotypes. These transformants all show characteristics of plants disturbed in meiosis: reduced fertility such as short siliques and low seed set.

### a. *DMC1* transformants

We made meiotic map from transformants with two different *DMC1* silencing constructs; RNAi-*DMC1* and Dominant Negative-*DMC1* (DN-*DMC1*). The cytological analysis of both transformants' pollen mother cells shows that meiosis is disrupted. As described by Couteau et al (1999), the DMC1 protein is needed for inter-homolog pairing and recombination (required for bivalent formation). The early prophase stage of both transformants (Figure 3.6a and j) showed that homologous chromosomes failed to

synapse as thin chromatin structures looping from the synizetic knot were observed. A fully synapse chromosomes were never observed. At later prophase, DAPI staining shows that there are more than five pericentromeric regions in both transformants (Figure 3.6 b & k). At diplotene, 10 separated chromosomes are visible (Figures 3.6c & l)



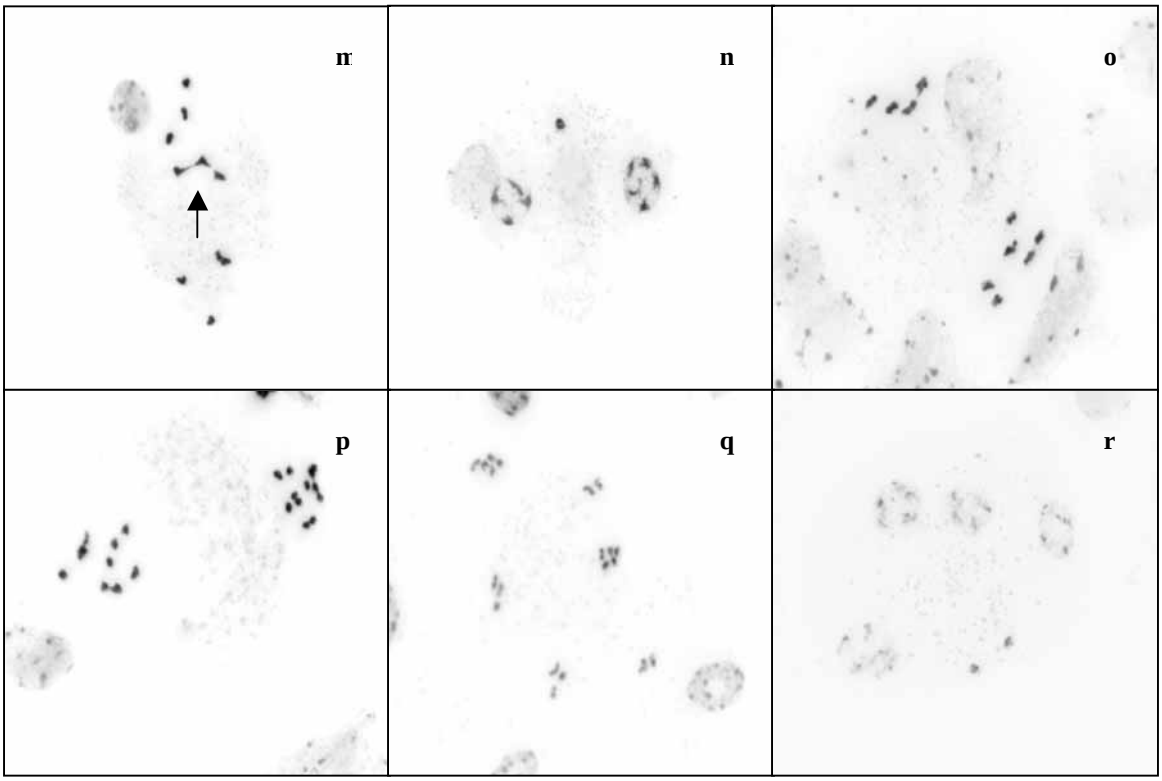



**Figure 3.6**: Meiotic atlas of two *DMC1* transformants prepared the same method as wild type. **a-i** shows RNAi-*DMC1* meiocytes and **j-r** shows DN-*DMC1* meiocytes. **a** Early prophase I cell, showing no synapsis. The presence of synizetic knot indicate zygotene phase. **b** Chromatin structure condense, and DAPI staining shows 10 pericentromeric regions. **c-d** Ten univalents in late diplotene and diakinesis stage. **e** Chromosomes starts grouping in the pseudometaphase I. **f** First meiotic division completed with organelle band in between, showing unbalanced dyad. **g-i** Erratic meiosis II products are prevalent in RNAi-*DMC1* transformant. **j** DN-*DMC1* transformant shows single strand chromatins in early prophase I. **k** In later prophase more than five pericentromeric regions are observed,. **l** Late diplotene, with some chromosome aggregation observed. **m** Arrow pointing at physical link between chromosome. **n** Dyad meiocyte with one lagging chromosome. **o** Dyad cell in metaphase II with 6-4 segregation. **p** DN-*DMC1* in anaphase II. **q-r** Polyad formation at the end of meiosis.

Ten independent univalents scatters throughout the cytoplasm in diakinesis stage (Figures 3.6 d, e, m). There were some residual cross-over between two chromosomes observed in DN-*DMC1* transforman (Figure 3.6m). The first meiotic division is completed when the 10 univalents organized themselves into subgroups separated by a cytoplasmic organelle band (Figure3.6f and n). Sometimes, lagging chromosomes were observed and these are grouped as cells with three, four, or five nuclei (Figures 3.6n). At metaphase II, aberrant chromosomes segregation is observed resulting in unequal chromosomes numbers in each

daughter cells (Figures 3.6 f and o) Meiosis II proceeds with the chromatids segregation in anaphase II (Figure 3.6p). In RNAi-*DMC1*, abnormal segregation is observed, producing daughter cells containing unequal number of chromosomes in each nucleus (Figures 3.6g, h, i). Figures 3.6 p and r show the end products of a second meiotic division in DN-*DMC1*. In both transformants, polyad formation or unbalanced tetrads are frequently observed. For example, six unbalanced gametes were produced from trinucleate cells (5-3-2 or 5-4-1) after the first meiotic division. After the second meiotic division, the chromatids in each group segregate into six nuclei. We also found that lagging chromosomes do not necessarily formed nucleus but also incorporate themselves randomly in other nucleus. This will be explained in the next subtopic (Subchapter III-b, Figure 3.11).

### b. *SDS* transformant

Figure 3.7 below presents some snapshots of meiocytes from pollen mother cell of transformants with an RNAi-*SDS* silencing construct. At early prophase, thin chromatin structures are looping out of the synizetic knot (Figures 3.7a). As the prophase I stage progresses, some parts along the chromosome show synapsis and some do not, distinguishable by thick and thin chromatin structure (Figure 3.7b). Azumi et al (2002) observed cells in pachytene stage, although in small percentage than the wild type, in the *sds* mutant. However, this is not observed in this transformant. At late diplotene (Figure 3.7c), 10 univalents are observed, with some univalents clustering together, possibly the NOR chromosomes. Chromosomes condense emphasizing the 10 univalents spreading around the cytoplasm, forming groups in pseudometaphase I. (Figure 3.7 d). At this stage, it is also observed that some univalents have a “V” shape structure, with the point

directing towards one pole. After the first meiotic division, homologs separate into groups divided by an organelle band. Segregation of univalents results in unbalanced daughter cells formation after the first meiotic division (Figures 3.7e-f). In some meiocytes, more than two groups are formed (Figure 3.7g). Following the aberrant meiosis I, second division follows with the production of nuclei with varying sizes (Figure 3.7h&i). Formation of polyads is also observed in the RNAi-*SDS* transformant.

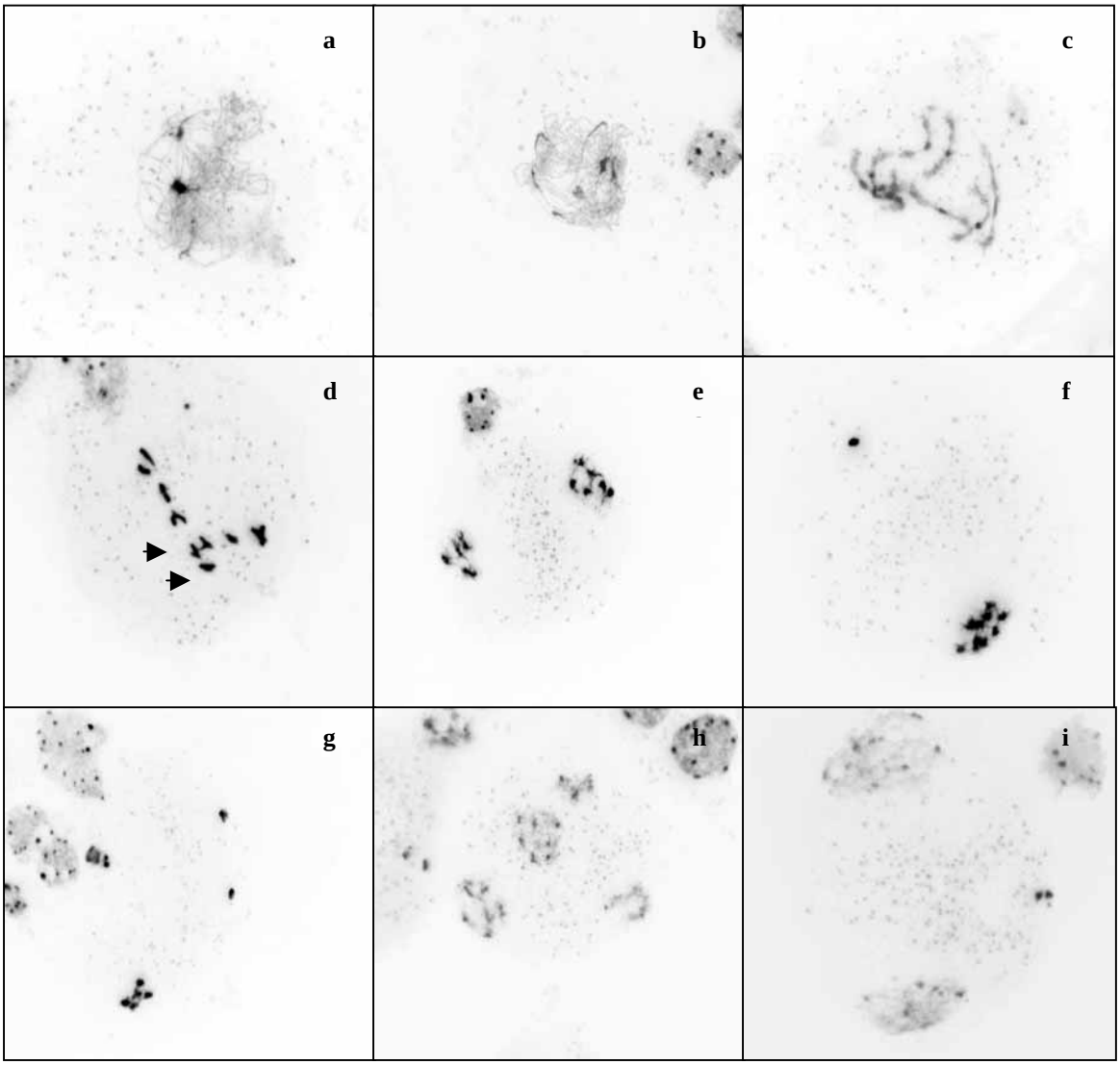



**Figure 3.7**: Meiosis in pollen mother cell of transformant RNAi-*SDS*. **a-c** Early prophase stage showing reduced pairing. **c**, Univalents are observed. **d** Condense chromosome structure reveals 10 univalents spread around the cytoplasm. Arrows point to "V" shape univalent, **e-g** Homologs separate and form two groups and some times more than two. **h-i** After meiosis II, 'tetrad' stage produces varying microspore sizes.

### c. *ASY1* transformant

The silencing of *ASY1* results in disrupted meiosis. The transformant shows failure to form complete synapsis in early prophase I, followed by a high numbers of unbalanced gametes at the tetrad stage. The meiocytes obtained from the pollen mother cells of this transformant are presented in Figure 3.8. The first stage shown is an early prophase I stage where thin stretches of chromatin structures loop out from the synizetic knot (Figure 3.8a). There are some short synapsed regions (Figure 3.8 b), but a fully synapsed chromosomes similar to the wild type pachytene stage are never observed. In diplotene stage, chromosomes seem separated but there are still some bivalents observed (Figure 3.8c). In pseudometaphase I, it is apparent that most of the chromosomes are completely achiasmatic (containing all univalents) or some cells with bivalents and several univalents (Figures 3.8 d, e). Ross *et al* (1997) indicated the mean bivalent frequency for *asy1* mutant to be 1.57 per cell so a low degree of synapsis is expected in the transformants. The first meiotic division shows irregular chromosome segregation in this transformant (Figure 3.8 f, g). At metaphase II, cells show unequal number of chromosomes and there are early segregating chromatids observed. These segregating chromatid are smaller in size compared to the segregating homologous chromosomes (Figure 3.8 g). At the 'tetrad' stage, this transformant forms nuclei containing varying numbers of chromatids inside each nucleus (Figures 3.8 h, i).

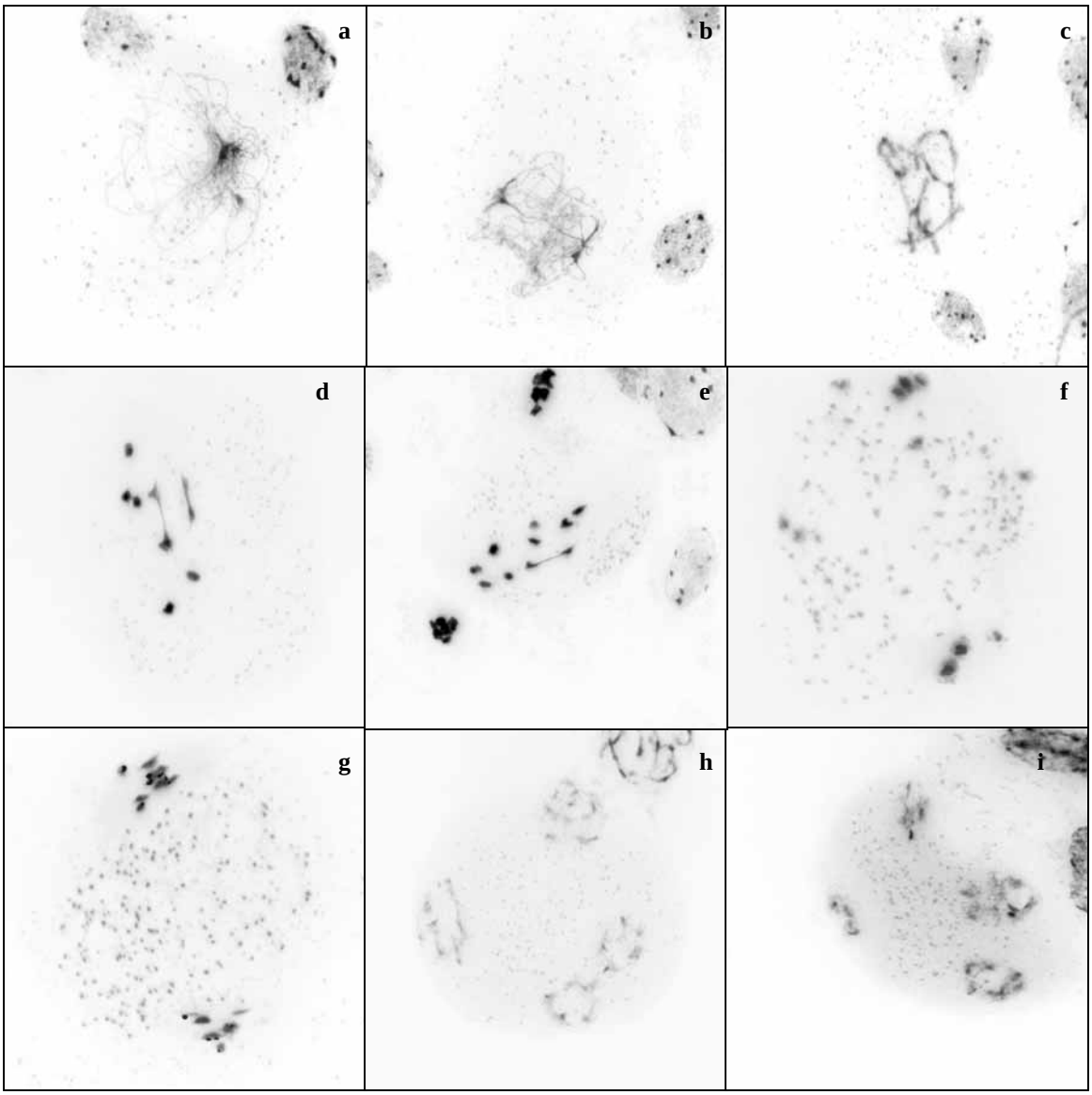



**Figure 3.8:** Meiotic map of RNAi-*ASY1* transformant. **a-b** Early prophase I showing unsynapsed chromosomes (a) and a cell with some short synapse regions (b). **c** Diplotene, showing most chromosomes in univalent and some bivalents **d-e** Pseudometaphase I. In d, there are two pairs of bivalents with six univalent and in e, one pair of bivalent with eight univalents are observed. **f** Dyad stage, showing two unequal nuclei and scattered chromosomes. **g** Metaphase II showing unequal chromosome groups and early chromatid separation. **h-i** tetrad stage, with unequal nuclei size at the end of meiosis.

## *III. FISH analysis and description of achiasmatic transformants*

In this part onwards, we only analyzed three transformants; RNAi-*DMC1*, DN-*DMC1* and RNAi-*SDS.*

### a. Products after achiasmatic Meiosis I

Images of dyad cells were captured, analyzed and classified in Excel spreadsheet. In all transformants, the chromosome segregation processes were disrupted, resulting in various nucleus sizes in dyad stage (Figure 3.9). From the descriptions of the behavior of these transformants during meiosis, it is observed that failure to form crossover, in other words, achiasmatic meiosis, leads to an unbalanced first meiotic division. In WT meiosis, five pairs of bivalents are observed, leading to the formation of a dyad containing 5 chromosomes (5-5) in each cell after the first meiotic division. However, absence or reduced bivalent in diakinesis stage was the main feature in all transformants. The absence of pairing is observed in the *DMC1* transformants but the DN-*DMC1* transformant shows some chiasma formation in the later prophase I stage. RNAi-*SDS* and RNAi-A*SY1* transformants show partial synapsis and RNAi-*ASY1* also has chiasma formation. Theoretically, at anaphase I, all 10 chromosomes have equal chance of either to migrate to the same pole or segregate to opposite poles. Therefore, it is expected that a range of dyad cells with 10-0, 9-1, 8-2, 7-3, 6-4 and 5-5 segregation at metaphase II follows the binomial distribution. However, the first meiotic division did not only produce dyad cells, but also meiocytes containing three and four groups of chromosomes. Table 3.1 summarizes the number of nuclei formed that are observed after first meiotic division.

**Table 3.1**: Nuclei formation after the first meiotic division

| | RNAi-*DMC1* | DN-*DMC1* | RNAi-*SDS* |
|---|---|---|---|
| 2 nuclei | 55 | 41 | 35 |
| 3 nuclei | 23 | 22 | 7 |
| 4 nuclei | 5 | 9 | 2 |
| 5 nuclei | 1 | 1 | 0 |
| 6 nuclei | 1 | 0 | 0 |
| total | 85 | 73 | 44 |

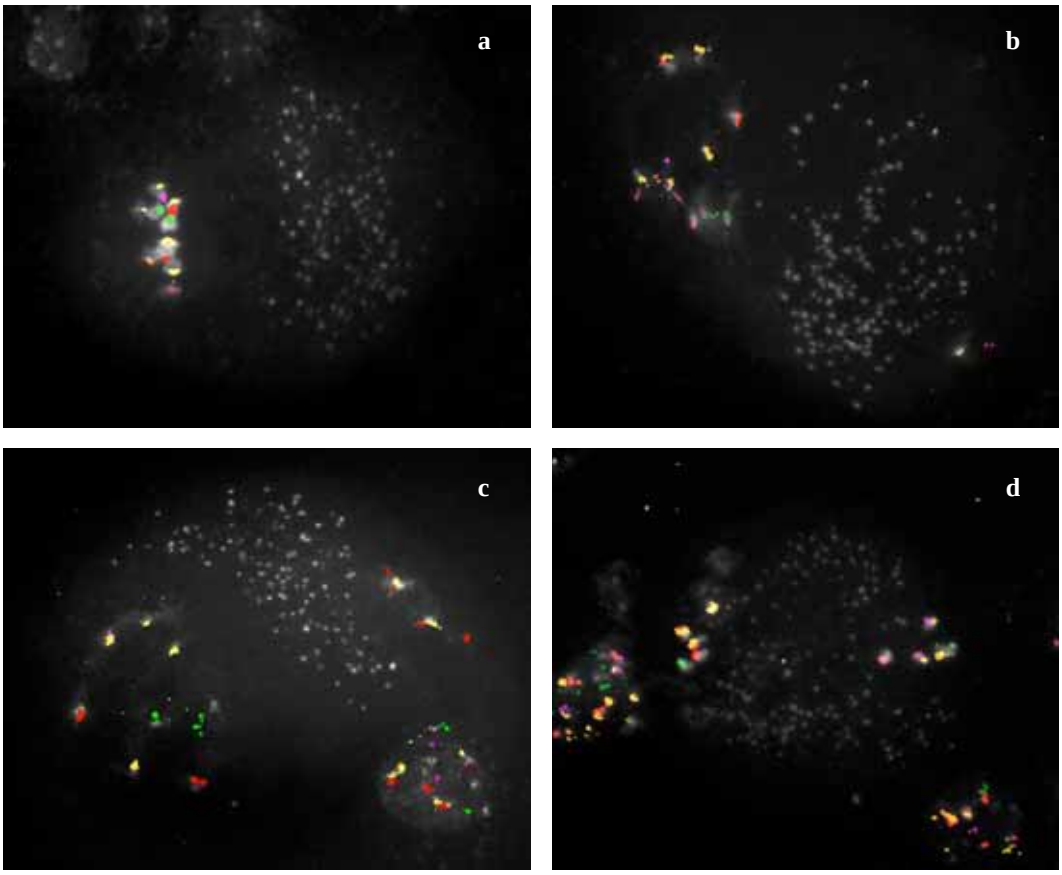



**Figure 3.9**: Dyad cells after first achiasmatic meiotic division. **a** Ten to zero segregation, observed in one of RNAi-*SDS* transformant. **b-c** Cells from DN-*DMC1* transformant **b** nine to one segregation **c** eight to two segregation **d** five to five (but unbalanced) segregation

Distribution plots for all dyad meiocytes (after the first meiotic division), which have nucleus sizes varying from 10 chromosomes to 0 chromosomes were made for all transformants and presented in Figure 3.10. The expected distribution for every nucleus size for these achiasmatic transformants is calculated using a binomial distribution curve

equation. In this case, there are 10 possible nucleus sizes. So, the probability of getting exactly nucleus size n is:

$$Exp = \left( \frac{10!}{n!(10-n)!} \times 0.5^{n} \times 0.5^{10-n} \right) \times p$$

Where there is a 0.5 probability of success (*n*) and a failure (10-*n*) after the first meiotic division. From 10 possible nucleus size, we are selecting nucleus size *n*. *p* is the total number of daughter cells produced after the first meiotic division for all transformants. For example, to get a nucleus size 10 (*n*=10) for the RNAi-DMC1 transformant (*p*=173), the expected frequency is $Exp = \left( \frac{10!}{10!(10-10)!} \times 0.5^{10} \times 0.5^{10-10} \right) \times 173$

$= 0.1689$

The expected distribution is compared to the observed nucleus size frequency for all transformants. For both *DMC1* transformants, the observed distributions of nucleus size are skewed to the right due to having a high number of nuclei with one chromosome (Figure 3.10, Graph a and b). The distribution of cell size for RNAi-*SDS* however shows an over-representation of nucleus with five chromosomes. This indicated that univalent segregation at anaphase I for these transformants was irregular, producing every possible nucleus size. Another distribution graph (Figure 3.11) was made for all transformants with meiocytes producing two daughter cells and non-aberrant meiocytes. A Chi-square goodness-of fit-test was conducted to see whether the observed distribution for nucleus sizes follows the binomial distribution or not (Table 3.2).

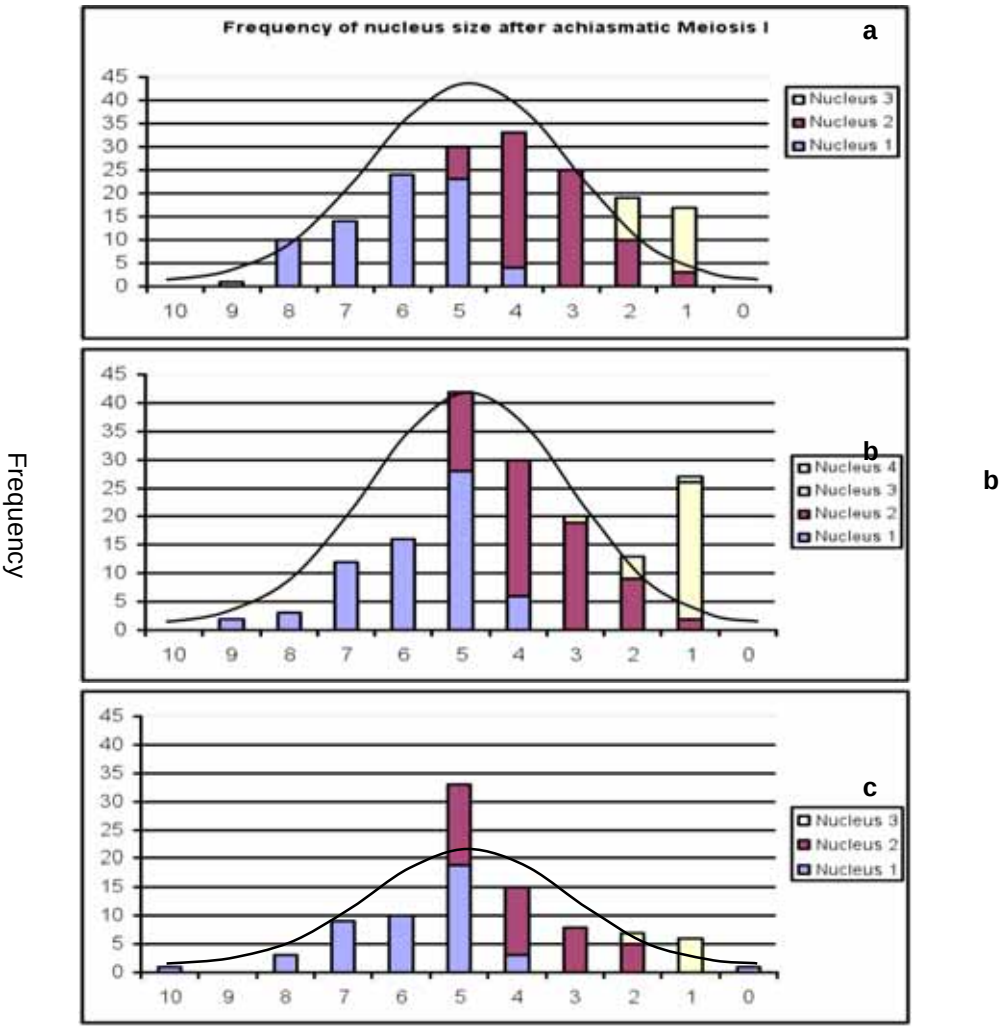



**Figure 3.10**: Distribution plot for chromosome content in nucleus (nucleus size) for all meiocytes after the first segregation for RNAi-*DMC1* (a), DN-*DMC1* (b), and RNAi-*SDS* (c). The legend indicate nucleus groups, from the nucleus containing the most chromosome (nucleus 1) to the least

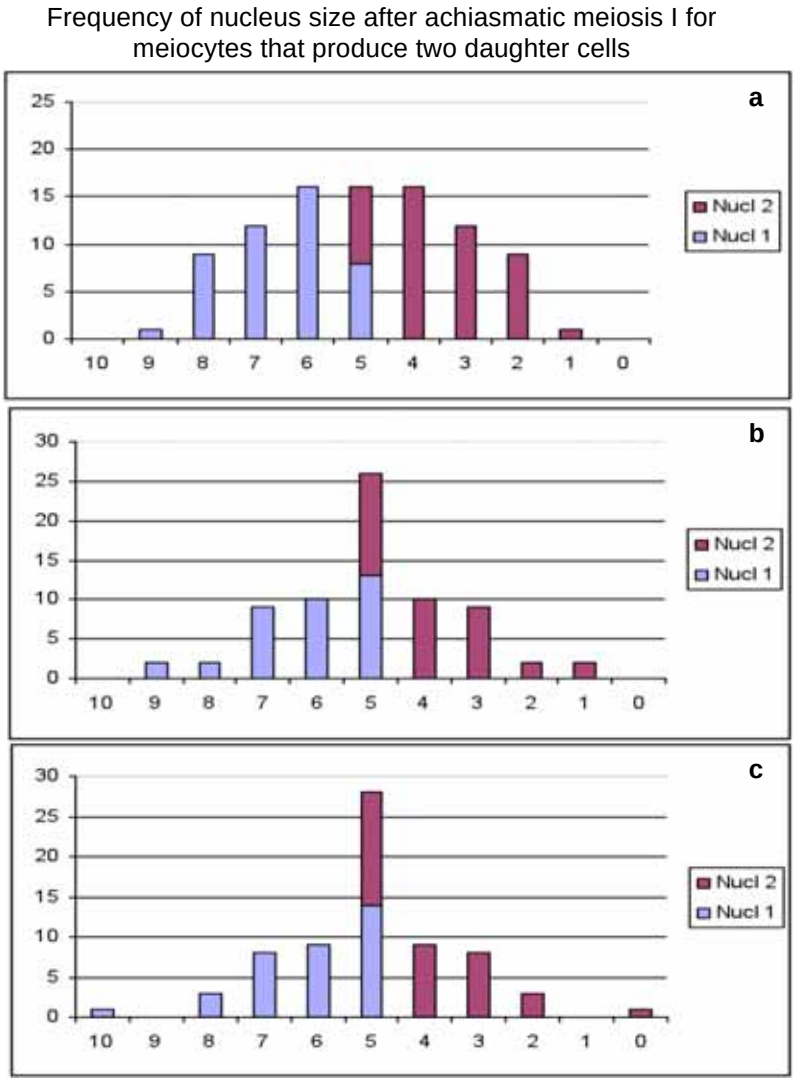



**Figure 3.11**: Distribution plot for binucleate meiocytes after first segregation for RNAi-*DMC1* (a), DN-*DMC1* (b), and RNAi-*SDS* (c). (Nucl 1, Nucl 2 = First and second nucleus respectively)

**Table 3.2**: Observed and expected frequency of nucleus size for binucleaate cells after Meiosis I

| Nucleus size | | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 | 0 | total | $X^2$ | p -value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Observed | 0 | 1 | 9 | 12 | 16 | 16 | 16 | 12 | 9 | 1 | 0 | 92 | 12.72 | 0.24 |
| RNAi-*DMC1* | Expected | 0 | 1 | 4 | 11 | 19 | 23 | 19 | 11 | 4 | 1 | 0 | | | |
| | Observed | 0 | 2 | 2 | 9 | 10 | 26 | 10 | 9 | 2 | 2 | 0 | 72 | 11.37 | 0.33 |
| DN-*DMC1* | Expected | 0 | 1 | 3 | 8 | 15 | 18 | 15 | 8 | 3 | 1 | 0 | | | |
| RNAi-*SDS* | Observed | 1 | 0 | 3 | 8 | 9 | 28 | 9 | 8 | 3 | 0 | 1 | 70 | 20.02 | 0.03 |
| | Expected | 0 | 1 | 3 | 8 | 14 | 17 | 14 | 8 | 3 | 1 | 0 | | | |

*p=0.05, degree of freedom = 10.*

Based on the analysis, the distribution of nucleus size for meiocytes with two nuclei of RNAi-*DMC1* and DN-*DMC1* transformants are not significantly different from the expected values. However, the RNAi-*SDS* transformant shows a high frequency of nucleus with five chromosomes. Having a high frequency of nucleus size five in achiasmatic meiocytes suggests that there is a higher chance of getting a cell with a regular disjunction. This is because an equally segregated dyad can only be among the nucleus with five chromosomes. Between the two *DMC1* transformants, DN-*DMC1* showed a high frequency of nucleus size five, but RNAi-*SDS* showed more nucleus size five than expected.

## b. Lagging chromosomes

In the cell size distribution plot (Figure 3.10), a high frequency of nucleus with one or two chromosomes is observed especially in the *DMC1* transformants. Figure 3.12 below shows example of meiocytes with lagging chromosomes. In all transformants, the frequency of nucleus with one chromosome (or lagging chromosome) is counted and shown in Figure 3.10.

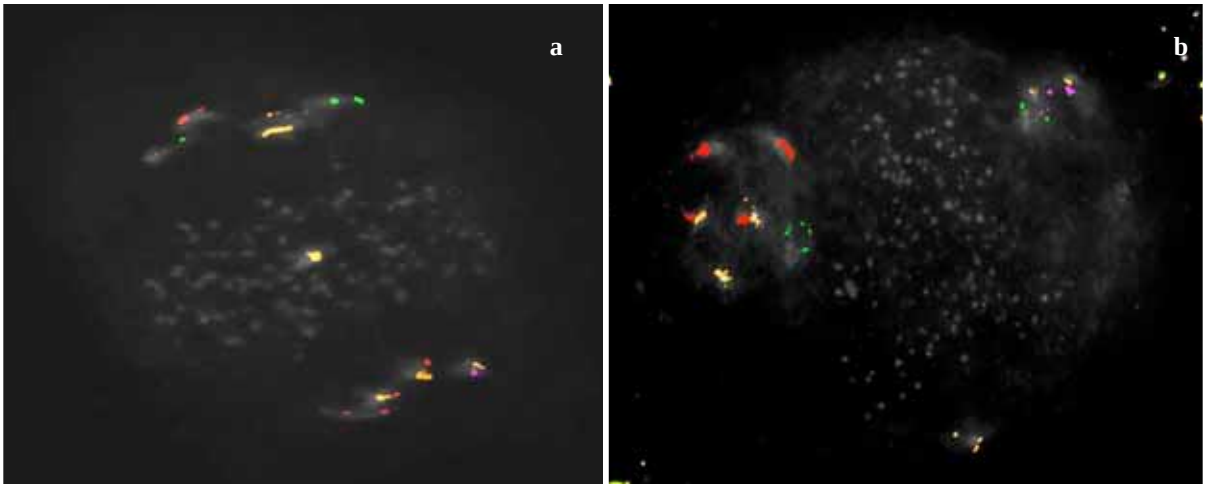



**Figure 3.12**: Three-group dyad cells tagged with FISH, showing lagging chromosomes

For RNAi-*DMC*1, 17 meiocytes have one of the daughter cells with one chromosome after the first meiotic division, 27 meiocytes for DN-*DMC1*, and six meiocytes for RNAi-*SDS*.

The observations of tetrad cells combined with the chromosome painting analysis reveals an explanation on the behavior of lagging chromosomes. In some tetrad cells of the achiasmatic transformants, it is observed that there are putative non-disjunctions of chromatids at meiosis II. Lagging chromosomes in achiasmatic meiosis are resolved in two possibilities; either forming a nucleus with one/two chromosomes (Figure 3.6r and 3.7i), or being incorporated randomly to other nucleus, as depicted in Figure 3.13. In the FISH image (Figure 3.12b) four perfect complementary nuclei are observed, with two

nuclei containing chromosomes 2, 3, 4 and 5 and the other two 1, 1, 2 and 5, as well as four single chromatids of chromosomes 3 and 4 (refer Figure 3.12a). The pink dashed arrow in Figure 3.13b shows the mechanism of regular equational division of chromatid in the second meiotic division. The incorporation of chromosomes 3 and 4 in this particular example complicates the regular mechanism of the second meiotic division, giving an 'unbalanced' equational division in the tetrad cells of these achiasmatic transformants. In achiasmatic meiosis, the fate of chromatids during the second meiotic division is equivocal.

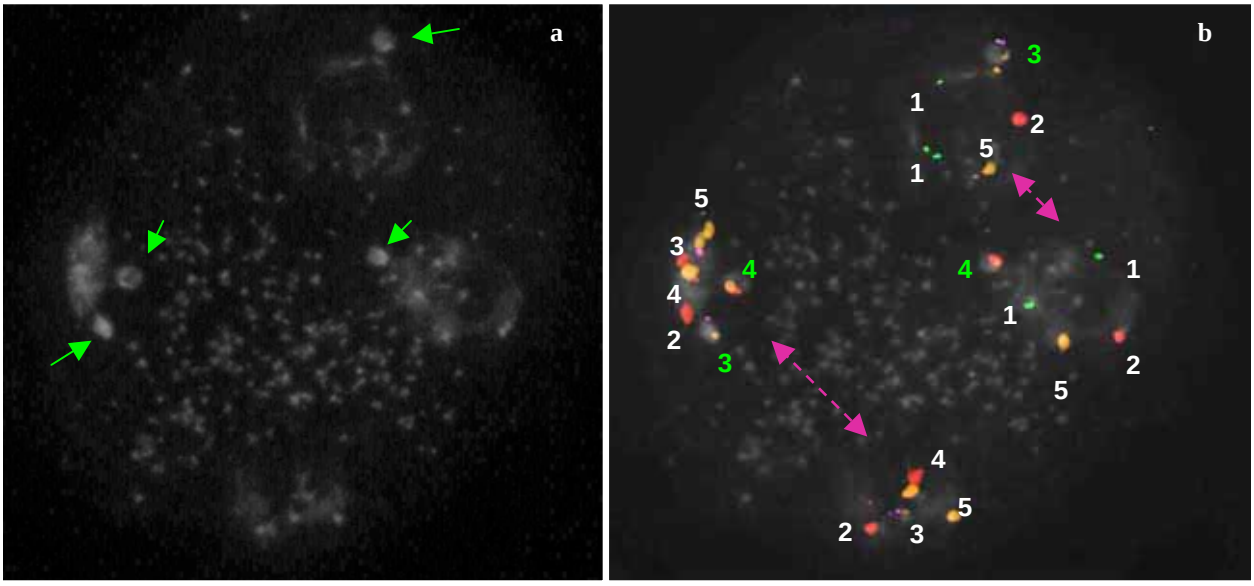
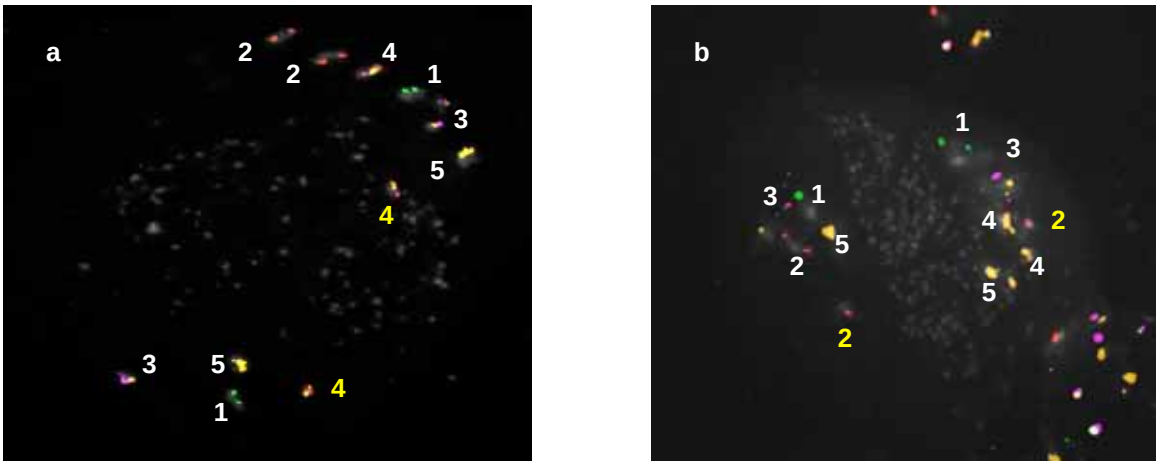



**Figure 3.13**: **a** DAPI image of four cell nuclei at the moment when the four sister chromatids join the larger cell (green arrow). **b** FISH labeled tetrad cell showing nuclei composition (equational division, depicted by pink dashed arrow) and joining chromatids (green number).

Therefore, analyzing tetrad cells with the understanding of the anomalies that happened in the previous stages (for example, the segregation of lagging chromosomes) gives a better insight on the chromosome behavior in achiasmatic transformants. The chromosome segregation analysis (Result III-d) is done on meiocytes after first meiotic division (dyad stage) instead of chromatid segregation in tetrad stage.

## c. Early chromatid segregation.

In WT meiosis, sister chromatids separate during the second meiotic division. On the other hand, in these transformants, we observed segregation of sister chromatids that happens during the first meiotic division, which will be referred as early chromatid segregation (Figure 3.14). This is mostly observed in RNAi-*DMC1* and DN-*DMC1* transformants Figure 3.15). Nevertheless, early chromatid segregation (ECS) was not observed in RNAi-*SDS* transformant. ECS occurrence for the transformants is 22% of the meiocytes (19/85) in RNAi-*DMC1* and 19% of the meiocytes (14/73) in DN-*DMC1* (data not shown). Chromosome 2 has the highest frequency of ECS (combining both *DMC1* transformants) whereas chromosome 5 has the lowest occurrence (Figure 3.15).



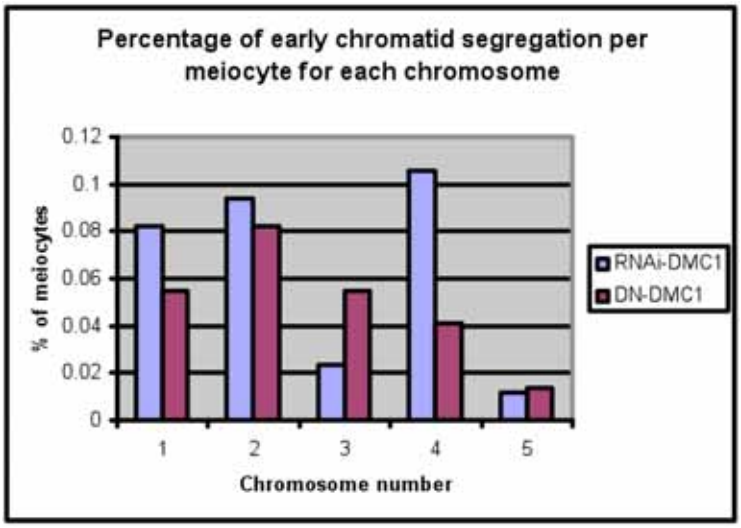




**Figure 3.14 (above):** Composite FISH image showing early chromatid segregation in **a** chromosome 4 and **b** chromosome 2 (yellow number).

**Figure 3.15 (left)**: The graph shows the percentage per meiocyte for every chromosome that undergoes early chromatid segregation.

## d. Chromosome segregation analysis.

As we have described before, two of the achiasmatic transformants, RNAi-*DMC1* and DN-*DMC1*, follow the random distribution as shown by the goodness-of-fit analysis for meiocytes yielding in two daughter cells after first meiotic division. The other transformant, RNAi-*SDS*, has a tendency to produce nucleus with five chromosomes. A chromosome segregation analysis after meiosis I was done on meiocytes with two daughter cells to see the percentage of regular disjunction or non-disjunction for every chromosome in these achiamatic transformants. However, a chi-square test is not able to be conducted because there is no information regarding the mean cell bivalent frequency for the transformants, which is needed to correct for the expected frequency of regular disjunction. We compared the percentage of regular disjunction and non disjunction for all five chromosomes in RNAi-*DMC1*, DN-*DMC1* and RNAi-*SDS*, summarized in Figure 3.16. Assuming randomness in segregation, an equal chance (50%) of all chromosomes to be properly segregating or having non-disjunction is expected. Based on Figure 3.15, the chromosomes in both *DMC1* transformants seem to have an equal chance to segregate regularly or not. For RNAi-*SDS*, all chromosomes (except chromosome 3) have an inclination towards having a regular disjunction after meiosis I. Nevertheless, this deduction still needs to be supported with a statistical analysis, which requires information on bivalent frequency of DN-*DMC1* and RNAi-*SDS* transformants.

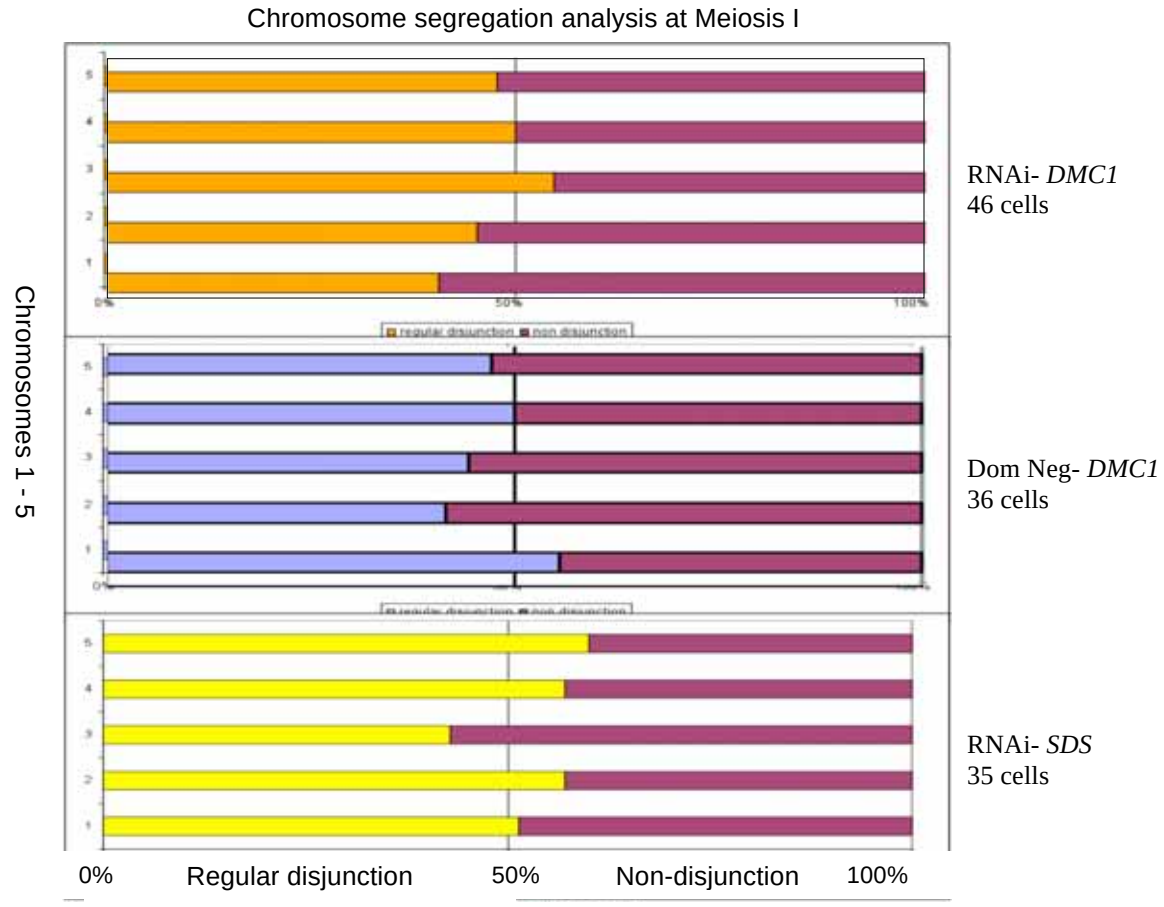



**Figure 3.16**: Percentage of each chromosome in all tranformants being non disjunction (right side) or regular disjunction (left side) for meiocytes in dyad stage with two nuclei**.**

# Chapter IV: Discussion and Conclusion

Comparison of the meiotic maps between the WT and the four transformants reveals that meiosis is irregular in the transformants. Cytological characteristics such as asynapsis at early prophase I, no chiasma formation in diakinesis, absence of or reduced bivalent formation, unbalanced segregation after first division, polyad formation or production of microspore with varying size are observed in all transformants. On top of that, all transformants show a sterile phenotype; low seed set and short siliques. The RNAi-*DMC1* transformant shows the same effect of the silencing of cross-over formation as the mutant phenotype as no chiasma is observed. Couteau *et al* (1999) described that *dmc1* mutant in *Arabidopsis* display a complete absence of chiasma. As a result, the chromosome segregation during the first meiotic division is irregular; culminating in the production of random nucleus sizes and low percentage of balanced daughter cells. On the other hand, the DN-*DMC1* transformant shows some residual cross-over. The DN-*DMC1* construct contains *DMC1* sequence without the N-terminal domain, which affect its DNA binding function (Kinebuchi *et al* 2005). The truncated DMC1 protein causes in the absence of male meiotic recombination in mice (Bannister *et al* 2007). In *Arabidopsis,* a dysfunctional DMC1 protein is expected to result in the silencing of cross-over formation, and thus, chiasma development. The residual chiasma observed in our DN-*DMC1* transformant might be due to the silencing of the construct in the plant. Even though the seedlings containing the construct are selected using a kanamycin-resistant gene (in the construct), some of the plants especially the DN-*DMC1* tranformant lost the sterility phenotype as they grew as they appeared to have wild-type phenotype (long

siliques, high seed set). Suppression of post-transcriptional gene silencing (PGTS) constructs were reported to be caused by callus induction and viral infection in potato and tobacco (Correa *et al* 2004 and Andrew *et al* 2000) but the mechanism of PGTS suppression in our case still needs to be studied.

The RNAi-*SDS* and RNAi-*ASY1* transformants also show residual cross-over but this is expected based on the description of their respective mutant phenotype. The *sds* mutant was described to have similar phenotype as *AtspoII* and *Atdmc1* mutant, although a fully synaptic pachytene stage was also observed in a low frequency in the *sds* mutant (Azumi *et al* 2002). Both the mutant and the transformant display some bivalent formation, but the frequency is not calculated for this report. The distribution pattern at anaphase I in the *sds* mutant exhibits 64.8% of binucleate meiocytes, nine of which have the distribution of 5:5 (Azumi *et al* 2002). In the transformants, out of 44 cells analyzed, 35 are binucleate meiocytes (79.5%), 14 of which have a 5:5 distribution. This shows that the *SDS* silencing has a less severe effect than the mutant.

The *asy1* mutant shows asynapsis in parts of the chromosomes at the prophase stage, followed by a low bivalent frequency (1.57) (Ross *et al* 1997). The bivalent frequency was not calculated for the RNAi-*ASY1* transformant but based from the meiotic map, it is expected for the transformant to have a higher bivalent frequency. This is because the RNAi-*ASY1* transformant showed some chiasmata formation at anaphase I, although this is not supported by any further numerical values and FISH analysis in our experiments.

Due to the improper segregation and unequal distribution in the first division the production of balanced gametes was expected to be low in these transformants, as exhibited by their respective mutants. For *Arabidopsis* with 5 basic number of

chromosome, there is only 0.03 chance ($1/2^5$) of producing balanced gametes after the random chromosome segregation. For RNAi-*DMC1*, 2 out of 84 (2%) cells in dyad stage were balanced meiocytes. This is considerably high for this transformants, considering that it displays many anomalies in meiosis. Nevertheless, this figure is obtained from the number of balanced meiocytes after the first meiotic division. Based on what was described in Figure 3.13, aberration in the second meiotic division could happen, resulting in unbalanced spores. For DN-*DMC1*, 4/80 (5%) dyad cells were balanced. RNAi-*SDS* produced 4 balanced dyads from 44 dyads analyzed (9%). The percentages for these two transformants are high because they showed residual chiasmata and have a tendency to produce a high number of meiocytes after meiosis I with both daughter cells containing five chromosomes.

It is also observed that a high number of nuclei with one chromosome, being the third/fourth group after first meiotic division. Based on our observation, we proposed that lagging chromosome can be resolved later in the second meiotic division as apart of a larger nucleus or develop a small nucleus, as explained in the result section (Figure3.13). With the chromosome painting approach, in all transformants, we observed tetrads in which nuclear composition suggests non-disjunction in meiosis II. The incorporation of non sister chromatids to the same nucleus in second meiotic division is similar to an irregular meiotic division known as parallel spindle. Parallel spindle in *Arabidopsis* is actually caused by a mutation in *AtPS1*, which produced first division restitution products at the end of meiosis (dyad instead of tetrads) (d'Erfurth *et al* 2008). In our case, the relation to parallel spindle lies in the fact that, resulting from an aberrant spindle

formation in meiosis II in these transformants, non sister chromatids can be incorporated in the same spore.

Asynaptic meiosis in these *Arabidopsis* transformants also produced early chromatid segregation (ECS). Study in maize proposed that a mature synaptonemal complex (SC) is essential for proper sister chromatid cohesion because asynaptic mutants in maize led to precocious chromatid segregation (Maguire 1990, cited in Roeder 1997) However, *zip1* mutant in yeast, which fails to develop SC, did not show ECS (Baker *et al* 1976). In the *sds* mutant of *Arabidopsis*, even though precocious separation of the arms of sister chromatid was observed in late prophase I, the subsequent division of sister chromatids seemed normal in meiosis II (Azumi et al 2002). The chromosomes of *SDS* transformant meiocytes in pseudometaphase I shows V-shape structure, displaying the same phenomenon as in the mutant. Nevertheless, no ECS are observed in the *SDS* transformant. ECS are observed only in the *DMC1* transformants. From the chromosome painting in *Arabidopsis* transformant, we found that chromosome 2 has a high tendency to undergo early chromatid segregation, followed by chromosome 4, whereas chromosome 5 has the lowest incidence of ECS. This suggests a preference in chromosome 5 to maintain sister chromatid cohesion through asynaptic meiosis. Nevertheless, we still need more information to confirm this. Based on the chromosome structure, we could say that ECS occurs more in acrocentric chromosome (2 and 4) and less metacentric chromosomes (1, 3 and 5). Studies in many organisms found that chiasma function depends of sister chromatid cohesion and showed terminal chiasma will be less stable (review Roeder 1997). Therefore, with the absence of chiasma in these transformants, the sister chromatid cohesion in acrocentric cohesion is less stable and

they would be prone to ECS. Another possibility of ECS preference in specific chromosome is the physical length. In *Arabidopsis*, chromosome 1 is the longest, followed by 5, 3, 2 and 4. Nevertheless the occurrence of ECS in chromosome 1 was considerably high compared to chromosome 5, so ECS does not depend on chromosome length. It could also be due to the presence of specific DNA sequence in the chromosome that helps to maintain the chromatid cohesion during meiosis I. Chromosomes with 5S locus (3 and 5) seemed to have low frequency of ECS. This suggests that a former chromosomes clustering event in the nucleolus. According to Ross et al (1997), chromosome clustering in the nucleolus was due to the aggregation of chromosomes with the NOR region (2 and 4) during late diplotene stage. We would expect these chromosomes to readily pair and form cross-overs. Contrary to this, chromosomes with 45S locus (2 and 4) have more occurrences of ECS. Combining the characteristic of chromosome 5 being metacentric, the longest and bearing 5S loci could explain the reason for more stable sister chromatid cohesion in chromosome 5.

Based on the analysis of chromosome segregation in binucleate products of these transformants, we made a segregation analysis to see the chance each chromosome being regularly segregated or being non-disjunction. In all transformants, we assume a 50:50 chance of having a regular disjunction (1:1) or a non-disjunction (2:0). A study by Pradillo *et al.* (2007) suggested that a proper formation of synaptonemal complex (SC) during early prophase, which leads to complete synapsis of chromosomes, will lead to proper chromosome segregation in the first meiotic division. They compared two asynaptic mutants (mutant that has defect in synapsis formation), *asy1* and *AtspoI-II* mutants, and one desynaptic mutant (mutant that has complete synapsis but unable to

maintain the connection until late prophase), the *dsy1* mutant. According to their findings, the chromosomes in the *dsy1* mutant has a higher chance of segregating regularly compared to the other two asynaptic mutants and they suggested that this is due to a proper formation SC in this mutant (*dsy1* showed wild type pachytene structure followed by having a significant tendency towards forming regular disjunction at meiosis I) (Pradillo *et al* 2007). In the description of the *sds* mutant, Azumi *et al* (2002) also shows that a low amount of meiocytes in the pachytene stage and high number of univalents in late prophase I, which suggest that *sds* is another desynaptic mutant. Figure 3.14 suggests that RNAi-*SDS* transformant has a tendency towards producing regular disjunction in almost all chromosomes although this is statistically not significant. Furthermore, .this is not supported by a sound statistical analysis because we miss the data for cross-over and bivalent frequencies, which is needed to correct the expected segregation frequency in the segregation analysis. Furthermore, we need more samples of the dyad binucleate cells to deduce a sound conclusion for this conclusion. It is interesting to see the segregation pattern of the *sds* mutant compared to the other desynaptic mutants to see whether the proposed function of SC from Pradillo *et al* (2007) still hold true for this mutant.

Based on the analysis, we can say that the RNAi-*DMC1* show a similar cytological phenotype as the mutant, but the DN-*DMC1* has some residual cross-over and this might be due to the silencing of the construct in some samples. Because of this, DN-*DMC1* transformant produces more nuclei containing five chromosomes and more balanced dyad cells than RNAi-*DMC1*. In the RNAi-*SDS* transformant, we did not notice any pachytene-like cells, as in the sds mutant description. In later prophase I and pseudometaphase, we observed early separation of the arms of sister chromatids in the

transformant, but this does not result in early chromatid segregation. RNAi-*ASY1* cells also shows similar phenotype as the mutant, such as having partial synapsis in early prophase I, precocious chromatid segregation, reduced bivalent frequency and unbalanced gamete production. However, it seems that the transformant has a higher bivalent frequency compared to the mutant, but no analysis is conducted to confirm this claim. All in all, the meiotic gene silencing gave similar effect as the mutants, although the silencing in some transformants is not complete.

The FISH analysis on the first three transformants reveals that chromosome segregation in *DMC1* transformants is random, producing various sizes of nucleus after the first meiotic division. However, the RNAi-*SDS* transformant produces a high frequency of nucleus with five chromosomes. Since a normally segregated and balanced nucleus can only be a nucleus with five chromosomes, having a high frequency of this increases the chance of getting more balanced gametes. Based on the analysis of binucleate dyads and the description of the anomalies in three of the transformants, RNAi-*SDS* is a reliable candidate for the applications of Reverse Breeding. It has a higher the chance of equal segregation in this transformant and shows a less severe meiotic aberrations, thus increasing the production of viable and balanced gametes that contain non-recombinant chromosomes. Nevertheless, this claim needs to be confirmed by further research. A five-colored FISH analysis using BACs for tetrad stage cells can be useful to get the accurate number of balanced gametes from these asynaptic transformants. The BACs on each chromosome must be located in tandem to ensure a more accurate and composited signal. We also need the information on bivalent frequency in RNAi-*SDS* to do a correction for the chromosome segregation analysis.

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# Appendix 1: Generation of Parental line from F1 hybrid by Reverse Breeding

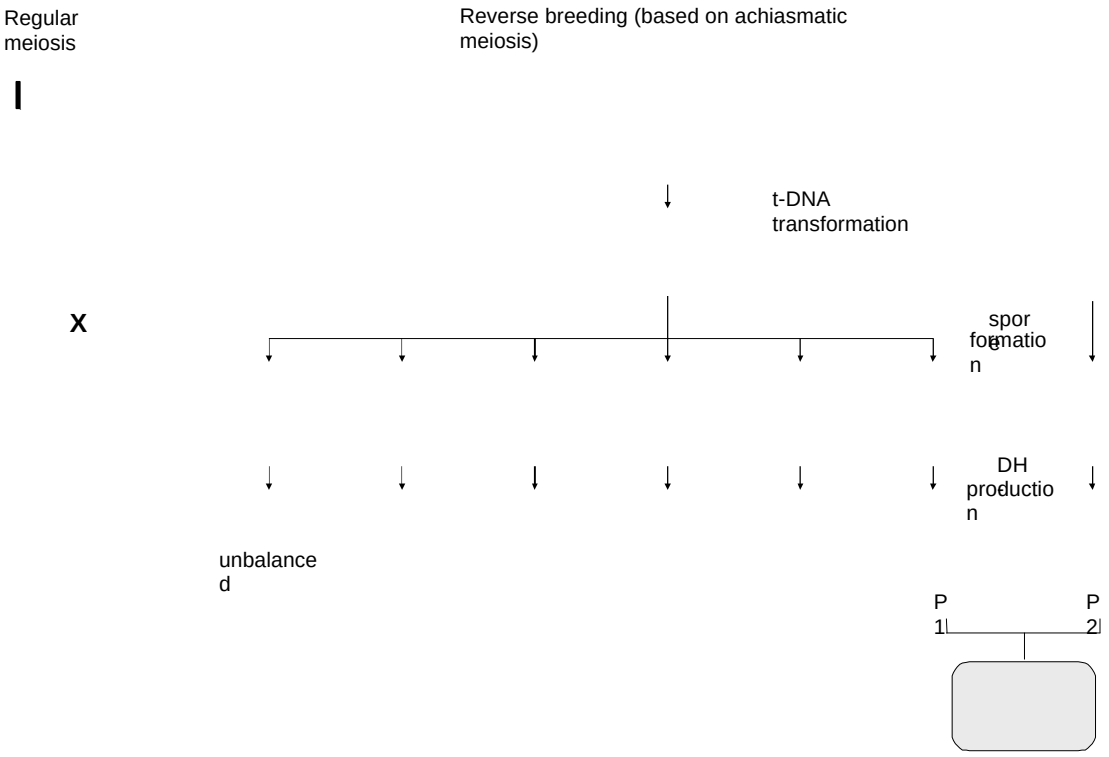
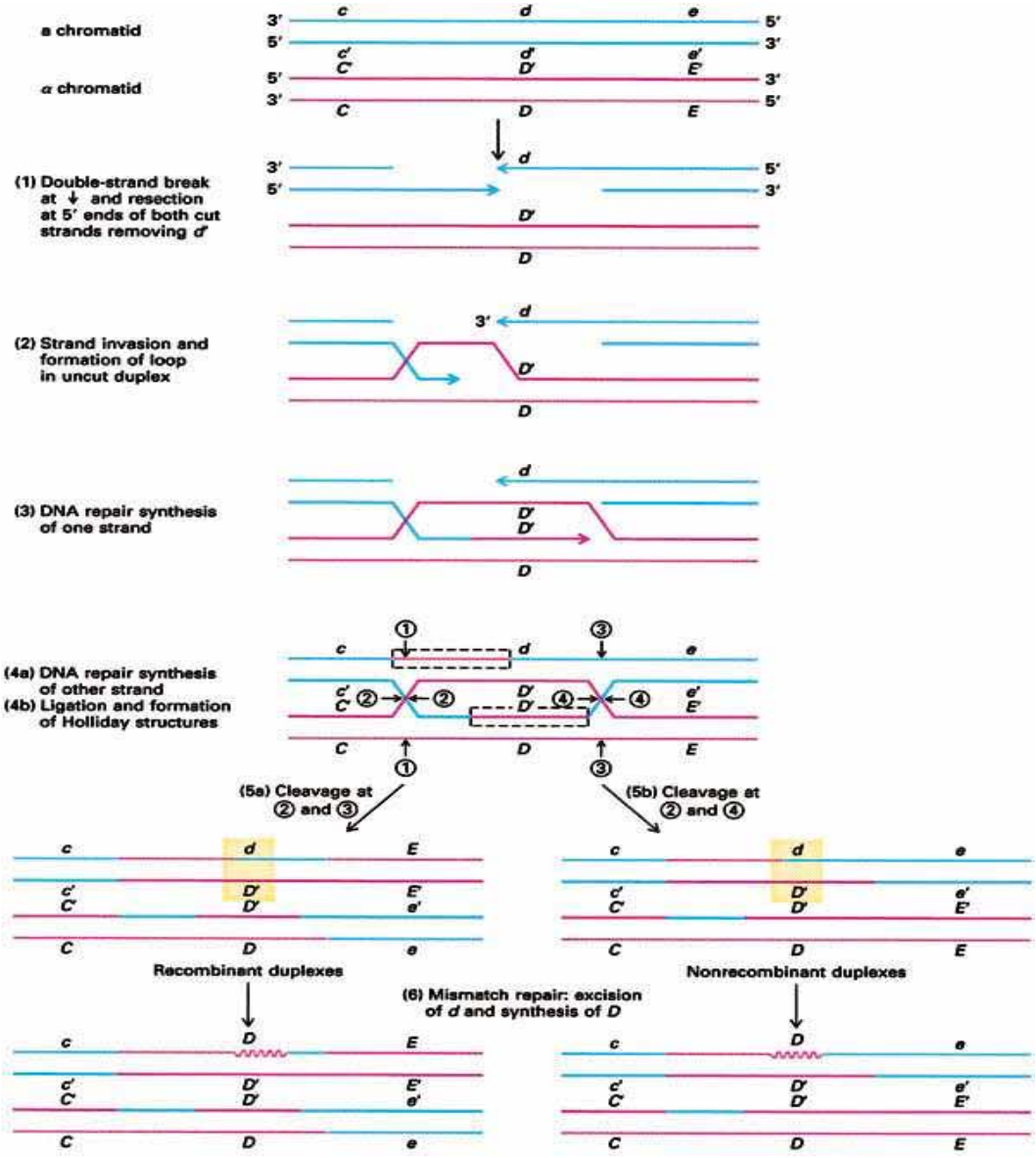



**Figure 1**: A scheme for Reverse Breeding for a hypothetical plant with basic chromosome number of 3 (taken from Wijnker and deJong 2008). Red dot shows the location of silencing construct.

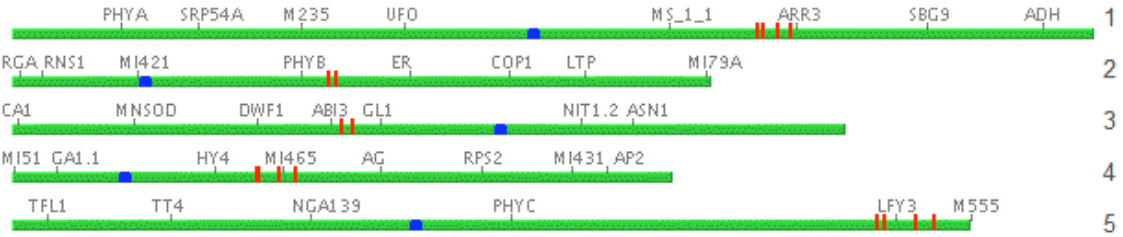
# Appendix 2: Double Strand Break Model




Source: www.nitro.biosci.arizona.edu

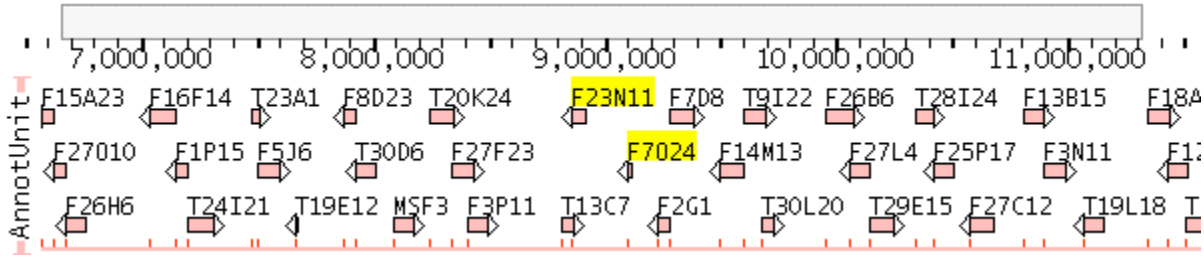
During meiosis, homologous chromosomes interact via recombination to produce at least one crossover (CO) per chromosome in order to segregate properly at the first nuclear division. Meiotic recombination begins with double strand breaks (DSBs) made by the endonuclease (a). As recombination progresses, joint molecules can be detected resulting from the strand invasion (c) being extended by DNA polymerization and then recaptured (d), creating a double Holliday junction that contains heteroduplex DNA (e). For each double Holliday junction, resolution is required to complete CO recombination and proper chromosome separation at the first meiotic division (f). DSBs can also give rise to noncross-overs (NCOs)

# Appendix 3: BAC information used in FISH experiment.

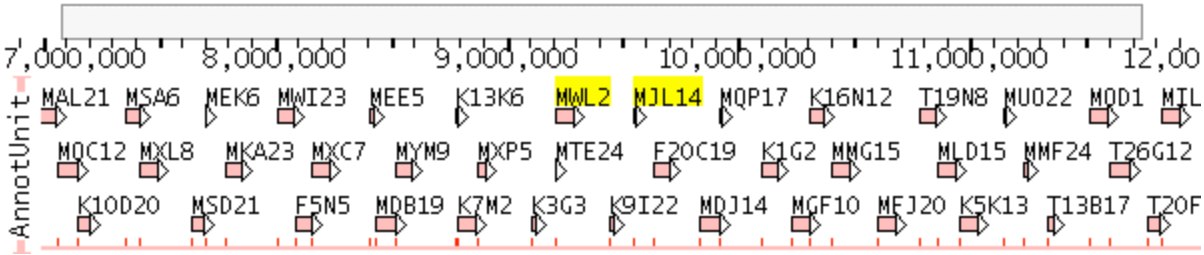


Overview of location for all BACs in all chromosomes

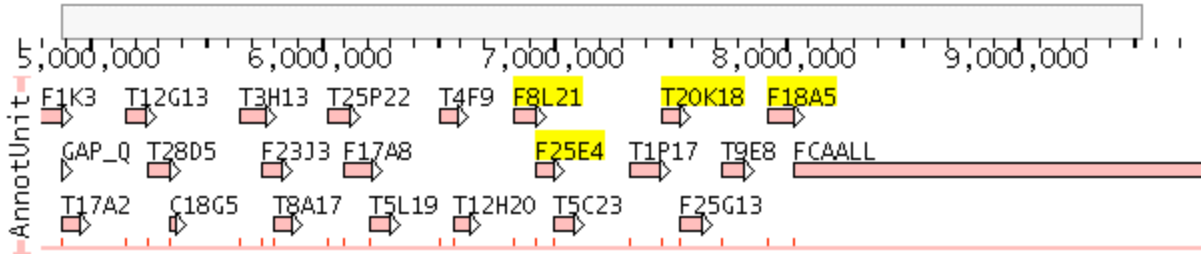
BAC location on Chromosome 1



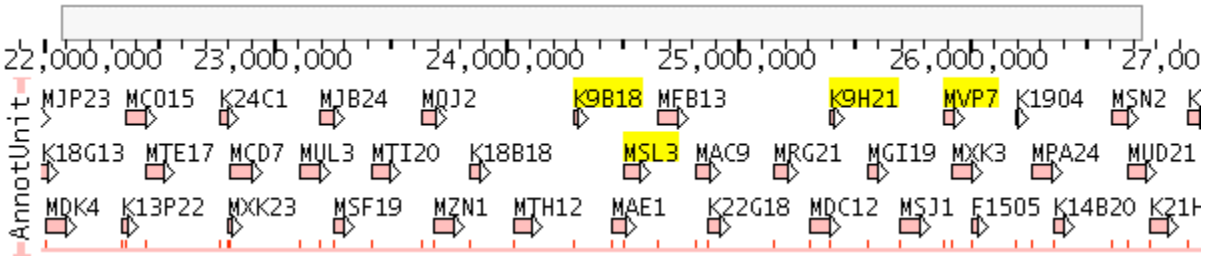
BAC location on Chromosome 2



BAC location on Chromosome 3



BAC location on Chromosome 4



BAC location on Chromosome 5

**Note**: All close-up view of BAC physical location was zoomed to 5MB

**Table 1**: BAC name and respective size in base pair

| Chromosome | BAC | Size (bp) |
|---|---|---|
| 1 | T6H22 | 96489 |
| | F13N6 | 90698 |
| | T18I24 | 85961 |
| | T30E16 | 100008 |
| 2 | F7O24 | 20078 |
| | F13N6 | 61384 |
| 3 | MWL2 | 84896 |
| | MJL14 | 10235 |
| 4 | F8L21 | 98948 |
| | F25E4 | 80019 |
| | T20K18 | 79375 |
| | F18A5 | 118718 |
| 5 | K9B18 | 20085 |
| | MSL3 | 71634 |
| | K9H21 | 25319 |
| | MVP7 | 49645 |

Source : http://www.arabidopsis.org/servlets/