Numerical and structural chromosome aberrations in cauliflower (*Brassica oleracea* var. *botrytis*) and *Arabidopsis thaliana*

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Chapter 1

General Introduction

Plant cytogenetics comprises the study of morphology and behavior of chromosomes and chromatin in the cell. It is a research field with a long history that dates back to 1842, when the Swiss botanist Carl Wilhelm von Nägeli described cell division during pollen formation (http://en.wikipedia.org/wiki/Carl_Nägeli). In the 20th century, major pioneers in this field were Cyril Darlington (http://en.wikipedia.org/wiki/C._D._Darlington), who discovered the mechanism of crossover formation, and Barbara McClintock (http://en.wikipedia.org/wiki/Barbara_McClintock), who described the structure and behavior of maize chromosomes. These general mechanisms were later described in many plant species including rice (Kurata et al. 1981, Cheng et al. 2001), sorghum (Magoon and Shambulinguppa, 1961), and tomato (Barton, 1950; Ramanna and Prakken, 1967). The plant genome is of remarkable plasticity and can show numerical and structural chromosome variants. The former group includes haploids, polyploids as well as all kinds of aneuploids, while the latter include deletions, duplications, inversions and translocations.

Numerical chromosome variants

Numerical variants may change gene balance and hence can profoundly affect morphology and fertility. Blakeslee (1922) characterized the globe mutant in Jimson Weed (*Datura stramonium*) and identified the presence of an extra chromosome in the cell complement (a trisomy). Later, Blakeslee (1934) described all different *Datura* trisomics, each of them containing a different extra chromosome and described the different morphologies of these plants and their seeds. Trisomies and other cases of aneuploidy were also found in other plant species as rice (Kurata et al. 1981), maize (Guo and Birchler, 1994; Makarevitch et al. 2008), wheat (Zhang et al. 2013), and *Arabidopsis* (Henry et al. 2005, Koornneef and van der Veen 1983).

Many plant species are diploids carrying two sets of homologous chromosomes like *Arabidopsis thaliana* (2n=2x=10), tomato (2n=2x=24), maize (2n=2x=20) and rice (2n=2x=24) (Anderson et al. 2010; Eckardt, 2008). A second major group of numerical chromosome variants are euploids that have one or multiple genomes (triploid, tetraploid, hexaploid etc.). Plants with only one set of chromosomes are called haploids or mono(ha)ploids, and because of their sterility hardly occur in nature. Haploids however have important uses in plant breeding practice where they are derived from (haploid) spores or gametes, or through uniparental genome elimination shortly after fertilization (Forster et al. 2007, Hermsen 1981, Kasha 2005). Haploids can double their chromosome number and so develop into homozygous diploid plants following somatic genome duplication. The resulting homozygous doubled haploids can then serve as new breeding lines or be used in mapping populations. Doubled haploids thus fulfill an important role in quickly obtaining homozygous plants from haploids cells.

When an organism contains three or more full copies of the entire set of chromosomes, it is considered a polyploid. There are two types of polyploidy: allopolyploidy and autopoly-

ploidy. Allopolyploids arise through interspecific hybridization and contain two or more related – but not identical – genomes (homeologues). Well known examples are *Brassica napus* (AACC), *Brassica juncea* (AABB), and *Brassica carinata* (BBCC), which are the allopolyploids formed from three diploid ancestors *Brassica rapa* (syn. *Brassica campestris*) (A genome), *Brassica nigra* (B genome) and *Brassica oleracea* (C genome) (U, 1935; Snowdon, 2007). Bread wheat is another famous example: the allohexaploid hybrid of three diploid species: *Triticum urartu* (AA genome), an unknown species related to *Aegilops speltoides* (BB genome) and an ancestral species *Aegilops tauschii* (DD genome) (Salse et al. 2008; Devos and Gale 2000). In contrast to the more common allopolyploids, there are limited numbers of natural autopolyploids. These have an intraspecific origin, and contain two or more sets of homologous genomes. Examples of these are potato (2n=4x=48) (Stupar et al. 2007), alfalfa (2n = 4x = 32) (Havananda et al. 2011) and the triploid and tetraploid sugar beet (2n=3x or 4x=27 or 36) (Savitsky, 1966).

Structural chromosome variants

The second class of chromosome variants includes deletions, insertions, inversions and translocations. These structural variants are numerous in the plant and animal kingdom and play an important role in evolutionary processes (Schranz et al. 2006, Lysak et al. 2007, Mandáková and Lysak 2008). When an organism is heterozygous for such rearrangements, this may lead to aberrant chromosome pairing, chiasma formation and chromosome transmission (resulting from inversion loops, translocation quadrivalents) as was extensively documented by Sybenga (1975). Such aberrant types may lead to decreased fertility and change the genetic transmission of traits (pseudo-linkage, hemizygosity). The consequences of these chromosome variants can consequently profoundly impact genetics and breeding programs (Sybenga, 1992).

In more recent years, major insights on the evolution of chromosome rearrangements and polyploidy, both between and within major crops, have come from the field of comparative genomics. The first large-scale comparative genome study focused on the major crop species in the family of grasses (Poaceae), in which rice (*Oryza sativa*), with its small genome size of 540 MB, was taken as a model. Other grass crops such as maize, sorghum and wheat were compared to this model, demonstrating both collinearity between specific chromosomes, but also the presence of (partly shared) genome rearrangements (Devos et al. 1993; Devos and Gale 2000; Devos 2005; Feuillet et al. 2002; Gale and Devos 1998; Moore et al. 1995; Nybom, 1954). Similar analyses, that also include chromosome painting (see further below), focused on groups of representative species from the *Brassicaceae* (Berr et al. 2006; Fransz et al., 2000; Mandáková and Lysak, 2008, Panjabi et al. 2008, Schranz et al., 2006, Wang et al., 2011). These demonstrated various (ancient) polyploidisation events, translocations and inversions. In the Solanaceae most of the comparative genomics involved species of the tomato clade (Aflitos et al., 2014). Also here, comparative chromosome painting

demonstrated the occurrence of structural rearrangements during the evolution of species within the genus *Solanum* (Szinay et al., 2012; Verlaan et al. 2011; Iovene et al. 2008; Lou et al. 2010).

Cytogenetic tools for plant research

Numerical chromosome variants and structural chromosome rearrangements are in many cases established during the description of the karyotype. Such a chromosome portrait displays the full set of chromosomes in a cell complement, often arranging the chromosomes in order of decreasing length and with centromeres on the same horizontal line. Each chromosome is defined by its morphology, *i.e.*, the length of the chromosome arms, its centromere position and further specialized regions like the nucleolar organizing region or NOR (which is the 45S rDNA domain, also called the secondary constriction) and heterochromatic banding patterns. In higher animals, chromosomes display such detailed banding patterns (G-banding) after chemical treatments or by the using of fluorescence dyes (Chen et al. 1986; Marks and Sehweizer 1974; Yunis and Sanchez 1973, Świtoński et al. 1996), thus allowing the identification of most or all chromosomes. In plant chromosomes such detailed G banding does not exist (Bickmore 2001). Instead, chromosomes are often small and morphologically very similar. Bands of constitutive heterochromatin (so called C bands) are in general confined to the pericentromere and NOR regions, thus limiting the number of morphologically identifiable chromosomes.

Chromosome identification of metaphase chromosomes

The lengths of long and short chromosomes, as well as centromere positions, are considered the two basic landmarks of chromosome morphology. Levan et al. (1964) proposed a chromosome nomenclature based on these characteristics. His nomenclature is now widely accepted for a large number of plant chromosome identifications (e.g., Ahmad et al. 1983). However, using this nomenclature, plant chromosomes in mitotic metaphase complements are mostly (sub)metacentric to acrocentric with little differences in size or centromere position prohibiting their morphological identification. Chromosome banding in plant can only be achieved through the C-banding technology. The C-banding was first applied on mouse by Pardue and Gall (1970), and then later successfully applied on plant species as rye, wheat, cucumber and triticale (Koo et al. 2005; Martin and Hesemann 1988; Seal and Bennett 1982). In most species though, the limited numbers of bands render C-banding relatively unsuccessful for chromosome identification.

Pachytene chromosome analysis

An alternative to mitotic metaphase chromosomes in plants for chromosome studies is that of pachytene chromosomes. These meiotic prophase I chromosomes are 10-50 times longer than their mitotic counterparts and also exhibit a fine structured pattern of heterochromatic blocks and chromomeres (de Jong et al 1999). Pachytene chromosome analysis is now considered a powerful method for chromosome identification and homologous pairing at meiosis, and is applied on most model and crop plant species, such as *Arabidopsis*, tomato, chickpea, brassica, pigeon pea and soybean (Fransz et al. 1998; Ahmad and Hymowitz 1993; Barton 1950; Dunhas 1983; Koo et al 2004; Singh and Hymowitz 1988).

Chromosomal *In situ* Hybridization

The experiments of Pardue and Gall (1970) not only demonstrated the existence of chromosome bands, as mentioned above, but were also one of the first successful attempts to hybridize 3H-thymidine labeled DNA sequences on chromosomes that were spread on microscope slides. The technology became widely applicable with the introduction of fluorescent labels and microscopes with epifluorescence in the nineteen eighties (Lloyd et al. 1989, Yoshii et al. 1995, Wilcox 1993, Wilkinson 1993, Komminoth et al. 1992). Nowadays the technique known as fluorescent *in situ* hybridization (FISH) became one of the most versatile tools in cytogenetics, allowing genomic, repetitive and single copy sequences to be visualized. Specialized methods were developed to distinguish parental chromosomes in interspecific hybrids (genome painting), to detect repetitive sequences on chromosomes and interphase nuclei (repeat painting) and mapping single copy DNA onto chromosome maps (single copy FISH) (Brooks 1993, Jin and Lloyd 1997, Koo et al. 2004, Pinkel et al. 1988, Speicher 1996, Cremer et al. 1988, Lichter et al. 1988, Popp et al. 1993). In plant studies such FISH techniques can be applied to metaphase chromosomes, interphase nuclei, pachytene chromosomes and even extended chromatin (de Jong et al. 1999, Schubert et al. 2001, Kato et al. 2011) and DNA fibers (Raap et al. 1996, Ersfeld 2004, Dai and Masatoki 2001, K. Wang et al. 2013).

Genome painting

Genome painting or genomic *in situ* hybridization (GISH) is one of the first widely applied technologies for the identification of parental chromosomes in interspecific plant hybrids (Schwarzacher et al. 1989; Sanchez-Moran et al. 1999). Genomic DNA of one species is then labeled and used as FISH probe onto target genomes. The technology was not only helpful in assessing the chromosomal composition of allopolyploid hybrids (Gill et al. 2009, Lim et al. 2007, Lim et al. 2007, Pendinen et al. 2012) or somatic hybrids (Collonniera et al. 2003, Escalante et al. 1998, Fu et al. 2004), but also helped to reveal the number of alien chromosomes and introgressions in backcross offspring (Kantama et al. 2007). In addition, GISH

can help to study (aberrant) chromosome pairing and recombination between homeologous chromosomes in offspring of wheat-rye hybrids (Benavente et al. 1996, Naranjo et al. 1987, Silkova et al. 2011) and *Brassica* (Han et al. 2003; Maluszynska and Hasterok 2005).

Repeat painting

Repeat painting is a FISH technology that uses repetitive sequences as fluorescent probes. Most of the studies on repeat distribution and organisation concern repeats of the 5S and 45S ribosomal DNA (Abd El-Twab et al. 2006, Abd El-Twab et al. 2012, Hasterok et al. 2001, Maghuly et al. 2010, Mantovani et al. 2005) as well as telomere repeats (Abd El-Twab et al. 2006, Armstrong et al. 2001, Bolzán et al. 2001, Scherthan 2002, Solovjeva et al. 2012). Increasing numbers of papers now appear on species specific satellite- and tandem repeats in heterochromatic blocks and centromeres (Koga et al. 2012, Lim et al. 2005, K-B Lim et al. 2007, Prakhongcheep et al. 2013), as well as dispersed repeats like LTR retrotransposons (Alix et al. 2005, Domingues et al. 2012, Salina et al. 2011) and microsatellites (Cuadrado et al. 2008, Danilova et al. 2014, Gallardo-Escárate et al. 2005, Schmidt and Heslop-Harrison 1996, Schneider and Molnár-Láng 2012). There are relatively few papers dealing with chromosomal positions of DNA transposons (Dimitri 2004, Sergeeva et al. 2010).

FISH with different repeats in a 2 to 5-colour mode enables colourful repeat maps that allow identification of individual chromosomes. Kato et al. (2004) applied this strategy on maize for the identification of mitotic chromosomes and the same strategy was applied on *Arabidopsis* (Fransz et al. 1998), tomato (Zhong et al. 1998) and wheat (Mukai et al. 1993). However, repetitive sequence painting may not always be efficient for chromosome identification since polymorphisms may exist between different varieties in the same species (e.g., Howell et al. 2002, K-Y Lim et al. 2007, Kato et al. 2004).

Single copy DNA FISH painting

Mapping single copy DNA sequences on chromosomes is of crucial significance in physical mapping studies. Probes can be obtained from different sized vectors like Yeast Artifical Chromosomes, YACs (Fransz et al. 2000, Liehr 2006, Zhong et al. 1999), Bacterial Artificial Chromosomes BACs (Fransz et al. 2000, Lapitan et al. 1997, Lysak et al. 2007, Szinay et al. 2008, Zhang et al. 2004), and even fosmids and plasmids (Dale 1985, Han et al. 2011, Wilschut 2009). By far most of the single copy-painting studies make use of BACs with insert sizes of 50 – 150 kb, large enough to produce strong fluorescent signals on a chromosome. The disadvantage of such large inserts is the presence of repeats, of which most are LTR retrotransposons which often produce many repeat signals over all chromosomes, mostly in the pericentromere regions (K-B Lim et al. 2007, Liu et al. 2008). Repeat signals can be suppressed by blocking with unlabeled genomic repeats, known as Cot-100 DNA (Paesold et al. 2012, Szinay et al. 2008, Tang et al. 2008). Alternatively, small single copy

sequences can be generated through PCR, which are then used as probes in FISH (Ma et al., 2010; S. Aflitos pers. comm.). Single copy FISH not only allows the identification of specific chromosome regions, but also can be helpful in analyzing chromosome behavior during meiosis, or to elucidate evolutionary relationship between related species, such as in potato and tomato (Verlaan et al. 2011; Tang et al. 2008).

Besides the non-FISH cytogenetic tool that I mentioned above (C-banding, chromosome nomenclature and pachytene chromosome analysis), the FISH technologies are considered most effective method for chromosome identification, but they depend on the availability of genomic or sequence information, or the presence of large insert vector libraries. In all other cases, karyotype analysis and chromosome identification depends on chromosome morphology heterochromatic banding patterns.

Flow cytometric analysis

Flow cytometry is an additional very useful tool to measure total DNA amounts of a eukaryotic organism (Herzenberg et al. 2006), but also helpful for demonstrating polyploidy

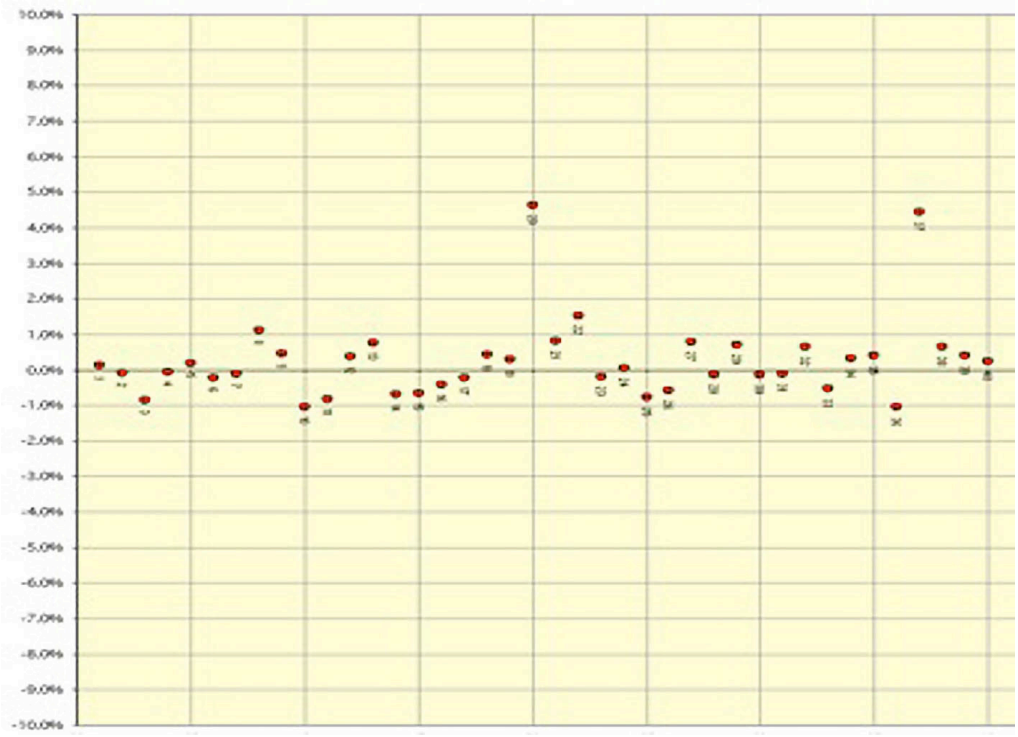

Figure 1. Aneuploidy in an arbitrary commercial sample of cauliflower plantlets as determined by flow cytometry (IribovSBW, Heerhugowaard, the Netherlands). Seeds were obtained from Chia Tai Seeds Inc., Kanchanaburi, Thailand, 2011. Seedlings with the numbers 20 and 37 have 5% more DNA, indicating the presence of an extra chromosome in their cell complements.

and aneuploidy in cells, tissues and seeds (Blanco et al. 2013, Cousin et al. 2009, De Laat et al. 1987, Pfosser et al. 1995, Roux et al. 2003). An example of the latter is shown in Figure 1, demonstrating the power of this technology to detect trisomics in a random sample of cauliflower plantlets. Although chromosome counting is more accurate, it requires expertise and is much more laborious than flow cytometry. The first study on plant cell flow cytometry was done by de Laat and Blaas (1984) in *Haplopappus gracilis.* In their report they describe how flow cytometry can be applied to plant cells to detect numerical chromosome variation and to identify chromosomes. Schwarzacher et al. (1997) applied flow cytometry to *Triticum aestivum* L. (wheat) cell lines to measure the DNA content of individual chromosomes. However, the strategy still has some technical difficulties as cell wall debris, the high chromosome similarity in most plant species and low metaphase content confound precise measurements (Doležel et al. 1994).

Numerical and structural chromosome aberrations in meiosis

The diagnosis of aberrant chromosome numbers and rearrangements is indispensible for explaining and/or predicting disturbances in meiotic pairing, chiasma formation, unbalanced transmission of chromosomes and sterility. This large field of cytogenetics is reviewed in numerous papers and was extensively discussed in Sybenga (1975, 1992). Here two aberrations are highlighted that are most relevant to this thesis.

Firstly, some chromosomes in cauliflower (*Brassica oleracea* var. *botrytis*) occasionally fail to pair and and/or form chiasma, which leads to the formation of univalents at the end of the meiotic prophase. This in turn leads to unbalanced chromosome segregation and aneuploidy among the offspring (Bodanese-Zanettini et al. 1983). Aneuploid cauliflower display aberrant phenotypes and pose a serious problem for breeders of this vegetable (Figure 2).

The second aberration that is to be highlighted is the paracentric inversion. This aberration can have profound consequences on meiosis and population structure. In a plant heterozygous for such an inversion, chromosome pairing may be absent in the inverted segment. In the case that a crossover occurs in the inverted segment, this leads to breakage of the chromosome pair at anaphase I. Either way in practice, recombination nearly never happens in a region harboring a paracentric inversion (except for the very rare case of gene conversion or the occurrence of a double crossover). The presence of a paracentric inversion therefore generally leads to a phenomenon known as linkage drag: the absolute linkage of several wanted and unwanted genes in a chromosome region. The occurrence of inversions not only plays an eminent role in chromosome evolution, but they are also important sources of problems in introgressive hybridization breeding programs.

Figure 2. Normal eudiploid cauliflower (left) and two aberrant cauliflower phenotypes resulting from aneuploidy (middle and left). The curds of the two aneuploid cauliflower plants are much smaller. Photo taken at the vegetable exhibition of the Royal Agricultural Station Angkhang, Chiang Mai, Thailand, 2014

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Contribution of genomics

Genomic information plays more than ever a crucial role in cytogenetic research. As for chromosome identification, *in situ* hybridization (especially BAC FISH) is considered an efficient technology. However, to get precise sequence information about probes, or to design specific PCR-based probes, genomic information of the plant species is required (Lysak et al. 2001, 2003; Zhong et al. 1998). For our study on chromosome identification in cauliflower (chapter 2 &3) we used genomic information about repetitive sequences to generate a unique repeat banding pattern on *Brassica oleracea* chromosomes. In addition, we designed a set of *Arabidopsis* probes for painting single copy regions in the genome of *Brassica oleracea* of which the larger part is triplicated. Using genomic information on the collinearity between the two species, it was possible to select specific *Arabidopsis* BACs to identify particular trisomies in cauliflower.

In our study on a paracentric inversion on chromosome 4 of *Arabidopsis*, we also greatly benefitted from DNA sequence information. Once FISH confirmed the inversion, the precise breakpoints could be established by comparing the full sequence information at the two breakpoints. In addition, comparative genomics of the inverted region in different accessions revealed unprecedented information on SNPs in and around the inversion.

Plant breeding aspects

The cytogenetic phenomena described in this thesis strongly involve disturbances in meiotic recombination, chromosome inheritance and genetic (in)stability and so are of pivotal importance for plant breeding. Instability of chromosome numbers in offspring may point to problems in meiotic pairing, chiasmata formation or balanced chromosome segregation at anaphase I/II. One of the best-studied meiotic genes in crop species is Ph1 in hexaploid bread wheat (*Triticum aestivum*). *Ph*1 is considered the major controlling gene for homologous pairing in this hexaploid crop (Griffiths et al. 2006, Holm and Wang 1988). In the absence of *Ph*1, chiasma formation between the homoeologues are no longer suppressed, such that all six homologues and homeologues now can associate and form multivalents and so may cause chromosome instability. The significance of this gene is that it allows homeologous introgressions, even with a less related species like rye. Plant breeders and geneticists therefore have a high interest to elucidate the underlying mechanism and to find comparable homeologous pairing regulatory elements in other species. A possible alternative to *Ph*1 is *PrBn*, a major gene controlling homeologous pairing in *Brassica napus* haploids (Jencyewski et al. 2003). Following the discovery of various meiotic mutants, it was shown in recent years that meiotic recombination and meiotic cell divisions to a considerable extent can be engineered to generate potentially profitable outcomes in breeding schemes. This is possible because of the intrinsic attribute of plant meiosis in which – contrary to other organisms – meiosis does not arrest when meiotic genes are mutated (Wijnker and Schnittger, 2013). The complete suppression of crossover recombination can induce chromosome inheritance, rather than the segregation of alleles as occurs in regular meiosis. This allows the recreation of parental lines directly from a heterozygous plant, when the non-recombinant parental homologues segregate to opposite poles in meiosis I and resulting gametes are subsequently regenerated as doubled haploids (Dirks et al., 2009; Wijnker et al., 2012). Alternatively, recombination can be threefold increased when the suppressor of crossover recombination FANCM is deleted (Crismani et al., 2012). The combined phenotype of three meiotic mutants was shown to turn a meiotic cell division into a mitotic-like division (d'Erfurth et al., 2009) and was shown to facilitate the clonal reproduction of hybrids through seeds (Marimuthu et al., 2011). The promises of meiotic engineering have been reviewed in Wijnker and de Jong (2008) and (Crismani et al., 2012)

Breeding programs in which alien chromatin (containing an economically desired trait) is introgressed in a recipient crop through introgressive hybridization, in few cases leads to linkage drag when the desirable trait cannot be recombined from the donor chromosome onto its recipient homoeologous counterpart. Identification of the region of interest may then be required using cytogenetic techniques, to understand the behavior of the chromosomal segment during meiosis and reveal the cause of this breeding problem. Linkage drag may have different causes. One is the localization of genes of interest in pericentromere heterochromatin, which is generally entirely devoid of crossovers. In a worse case scenario, the

desirable genes are located in structurally rearranged chromosomal regions like paracentric or pericentric inversions that prohibit crossover recombination between homeologous chromosomes. In this case there is no possibility to break the pseudo-linkage between the trait and its flanking undesirable genes (Canady and Chatelat 2006).

Focus of this thesis

In this thesis, I used cytogenetic tools to study numerical and structural chromosome aberrations in *Arabidopsis thaliana* and cauliflower (*Brassica oleracea*). In *B. oleracea*, especially in cauliflower varieties, there is a serious problem of meiotic instability leading to aneuploid offspring with aberrant phenotypes (Figure 2). I optimized and applied different painting technologies to identify the chromosome(s) causing aneuploidy. I also analyzed different stages of meiosis to elucidate the course of chromosome missegregation, and compared these observations with meiotic disturbances in the *Ap1*/*Cau* double cauliflower mutant in *Arabidopsis*. In *Arabidopsis thaliana* different accessions were investigated for the presence of a heterochromatic knob in the short arm of chromosome 4 resulting from a paracentric inversion on this chromosome arm. Twenty accessions were screened for the presence of this knob in pachytene complements, and these cytological data were compared with a PCR-based analysis for the presence of the inversion using primers around the inversion breakpoints.

Chapter 2

In this chapter, I describe the construction of a general karyotype for cauliflower based on repeat painting. To identify all chromosomes in the cell complement, a 5-color FISH assay is presented, using 5S rDNA, 45S rDNA, and three LTR retrotransposons as probes. I also will discuss the advantages and drawbacks of this repeat painting technology.

Chapter 3

Here a cauliflower karyotype is presented based on cross species painting using *Arabidopsis thaliana* BACs as probes. Since *Brassica* underwent a genome triplication since its divergence from *Arabidopsis*, each BAC gives up to six foci on a diploid cell complement of cauliflower. In order to generate unique FISH patterns for chromosome karyotype of *B. oleracea* (since repetitive sequences may have polymorphism in different varieties of *B. oleracea*), I combined probes from different BACs using mummerplot technology comparing the genomes of *Brassica oleracea* and *Arabidopsis thaliana*. This probe set of differentially labeled BAC pools allows the unequivocal identification of all different chromosomes in the cauliflower chromosome. It is shown that the probe set allows the identification of trisomics and telotrisomic in cauliflower.

Chapter 4

This chapter describes the behavior of *Brassica oleracea* chromosomes during different meiotic stages to pinpoint the cause of the unbalanced chromosome segregation that in this species generates trisomics in its offspring. Large numbers of univalents were found to be

formed at diakinesis / metaphase I, in spite of normal chromosome pairing at pachytene. Meiosis in cauliflower can be characterized as partial desynaptic. Interestingly, a reduced number of MLH1 foci at diakinesis is observed, suggesting that crossover numbers are reduced. To test as to whether cauliflower phenotype in *Arabidopsis* is also suffering from desynaptic meiosis we also analyzed pollen mother cells of the *AP1*/*Cau* double mutant.

Chapter 5

This chapter describes different aspects of a paracentric inversion on *Arabidopsis* chromosome 4. This paracentric inversion relocates a portion of the pericentromere heterochromatin onto the short arm of chromosome 4, which is visible as a heterochromatic knob. With the use of BAC FISH onto the cell complements of an inversion heterozygote, the inverted orientation of this chromosomal region on the homologs is demonstrated. Using immunofluorescence microscopy with antibodies against ASY1 and ZIP1, that respectively stain meiotic prophase chromosome axes and the synaptonemal complex, it is shown that the homologous chromosomes in this inverted region do align, but do not synapse. By visually verifying the presence or absence of the heterochromatic knob in 20 accessions, the reliability of a PCR-based method is shown that allows testing the presence or absence of the heterochromatic knob (and the paracentric inversion) using primers spanning the inversion breakpoints.

Chapter 6

In this general discussion the major conclusions of the research in my thesis are revisited to provide a synopsis of the major advantages and drawbacks of cytogenetic technologies. I will especially shed light on the potential of meiotic analysis, advanced FISH technology and flow cytometry for plant breeding purposes.

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Chapter 2

FISH painting with repetitive DNA sequences for chromosome identification in aneuploid cauliflower (*Brassica oleracea* L. var. *botrytis*)

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Abstract

A common problem in cauliflower (*Brassica oleracea* L. var. *botrytis*) cultivation and breeding is the occurrence of high numbers of aneuploid offspring with aberrant phenotypes. To reveal which chromosomes cause aneuploidy, a cytogenetic tool is required to distinguish the different cauliflower chromosomes. Since mitotic chromosomes in cauliflower are morphologically rather similar, we developed a karyotyping tool based on Fluorescent *in situ* Hybridization (FISH) that allows clear identification of all *B. oleracea* chromosomes using repetitive sequences. This repeat painting is a five-color FISH using five different repeats: 5s rDNA, 45S rDNA, two *Brassica rapa* centromere-specific repeats and a *B. rapa* BAC (KBrH092N02) containing a dispersed repeat of unknown class. Using this painting scheme all *B. oleracea* chromosomes could be identified unequivocally in mitotic and meiotic cell complement, as well as extra chromosomes or chromosome arms in aneuploid cells. The robustness of the method for karyotyping other *B. oleracea* crops using repetitive sequences is discussed.

Introduction

Cytogenetic analysis of a eukaryotic organism generally begins with the morphological description of its chromosomes, known as chromosome portraying or karyotype analysis. This describes the lengths, centromere positions and banding patterns of the chromosomes in a cell complement (http://en.wikipedia.org/wiki/Karyotype, and references therein). This was done for countless numbers of species including human (Pearson 1972), dog (Yang et al. 2000), rice (Cheng et al. 2001; Ohmido and Fukui, 1995), wheat (Gill et al. 1991; Gerlach 1977), tomato (Ramanna and Prakken, 1967), soybean (Findley et al. 2010). Karyotype analysis allows the construction of physical chromosome maps, that in turn helps in the assembly of genomic DNA sequences, supports genetic mapping and can provide insights into phylogenetic and evolutionary relationships between species (Adams et al. 2000; Chamala et al. 2013; de Fátima Ruas et al. 2005; Jiang et al. 1995; Mandáková and Lysak 2008; Shearer et al. 2014; Stack et al. 2009; The Tomato Genome Consortium 2012).

In the early decades, chromosome arm lengths were used to describe the karyotype, in which the lengths of the chromosome arms were measured, and chromosome arm ratios were calculated to identify chromosomes (Levan et al. 1964). However, this strategy may not be sufficiently accurate and reproducible, as for many species, such as most of the *Brassica* crops, the chromosome arm ratios in the cell complement are almost identical, so that even sophisticated digital measurements could not help much with unequivocal identification of chromosomes.

Chromosome banding through chemical or physical treatment of slides with spread chromosome can help the process of identification, provided that unique banding patterns can be obtained for different chromosomes. Different banding techniques like C-banding,

N-banding and modified C-banding were successfully used for the description of different chromosomes in wheat, rye and cucumber (Gill et al. 1991; Gerlach 1977; Martin and Hesemann 1988; Seal and Bennett 1982; Koo et al. 2005). A more recent technology uses fluorescent probes in fluorescent *in situ* hybridization (FISH) experiments on spread chromosomes to generate unique banding patterns in different colours to distinguish chromosomes in cell complements for karyotyping. FISH technology is more informative, because it identifies regions of known sequences (*i.e.* the probe sequences) rather than unknown sequences. It is also more versatile and reliable in comparison to simple karyotype analysis with or without chromosome banding.

Different DNA sequences can be used as hybridization probes. Total genomic DNA can be used as probe for genome painting, in which parental chromosomes can be distinguished in a sexual or somatic interspecific hybrids (Markova and Vyskot, 2009; Silva and Souza, 2013). A second class involves repetitive sequences, like the ubiquitous ribosomal DNA (rDNA) and telomere repeats, satellite / tandem repeats, or dispersed retrotransposons. Such repeats generate fluorescent foci on one or several pairs of chromosomes and have been used in most crop and model species, including *Arabidopsis thaliana* (Koornneef et al., 2003), maize (Kato et al., 2005; Lamb and Birchler, 2006), *Pinus* species (Hizume et al., 2002) and Norway spruce (Vischi et al., 2003). The most commonly used repeats for chromosome identification and karyotype analysis through fluorescent *in situ* hybridization are rDNA, centromere-specific and telomere repeats (Kato et al., 2005). A third class of DNA sequences comprises unique and single copy sequences, the use of which will be further discussed in Chapter 3 of this thesis.

Chromosome identification and karyotype analysis in *Brassica* crops has been challenged for decades due to the lack of chromosome specific features in mitotic and pachytene cell complements. Several banding and FISH technologies were adapted for use in these species and include C-banding (Olin-Fatih and Heneen 1992, Olin-Fatih 1996), Chromomycin A3, 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining (Fukui et al. 1998), and FISH with rDNA, centromeric tandem repeats and pericentromeric tandem repeats (Chiang et al., 1979; Armstrong et al., 1998; Howell et al., 2002; Lim et al., 2007; Xiong and Pires, 2011). Making use of several repeat sequences, it was possible to identify the nine chromosome pairs of *Brassica napus* (Xiong and Pires, 2011).

Our interest in chromosome identification in cauliflower (*Brassica oleracea* var. *botrytis*) has a special reason. The crop shows high numbers of aneuploids in its offspring, most of which are trisomics (unpublished results). The occurrence of trisomics is highly problematic as these aneuploids show deviant phenotypes and decrease yield. Aneuploidy is not uncommon among plants. Blakeslee (1921) was the first to describe this numerical chromosome aberration in Jimson weed, *Datura stramonium*. After his pioneering work, many more examples of aneuploid plants were described, e.g. maize, tomato (Weber 1983; Makarevitch and Harris, 2010; Khush and Rick, 1966). Aneuploids are more often found in polyploid species, such as wheat (Henry et al. 1996, Zhang et al. 2014), where meiotic

segregation is less often balanced but where their occurrence is less problematic as these aneuploids have smaller effects on viability and fertility (Comai 2005, Doyle 1986, Papp et al. 1996). Although aneuploids also occur in animals, their occurrence is far less common, as these organisms are much more sensitive to gene dose changes (Torres et al. 2008). In most mammals, aneuploidy for most chromosomes is lethal except for the sex chromosomes (syndromes of Turner, Klinefelter, Jacobs). In human only few cases of viable aneuploids have been described of which the patients show different phenotypes, like the Patau- and Down syndromes (Antonarakis et al. 2004, Luthardt and Keitges 2001).

Aneuploidy may cause chromosome structure instability and leads to aneuploidy formation in the offspring, since it unbalances highly balance-sensitive component as spindle which leads to destabilize the symmetric chromosome segregation during meiosis, and changes of gene expression, which may lead to phenotypic changes (*e.g.*, Duesberg et al. 1999, Huettel et al., 2008; Matzke et al., 1999; Makarevitch et al., 2008; Henry et al. 2010), and may even lead to sterility. Chromosome loss may be even worse than having extra copies of a chromosome. In maize it was found that monosomes (plants missing one chromosome) were completely sterile. Conversely, the allohexaploid bread wheat is highly tolerant for missing (or having extra copies of) chromosomes, and can even tolerate complete nullisomy (in which one of the homologous chromosome pairs is absent). Meiosis in trisomic plants leads to specific problems: the three chromosomes cannot segregate in a balanced manner, leaving two chromosomes to segregate to one pole and one to the other. In other cases an unpaired chromosome (univalent) may lag behind on the equatorial plane and gets lost or forms micronuclei (Khazanehdari and Jones, 1997). Alternatively, the univalent breaks at the centromere, producing single arm telosomes (Sybenga 1992). Although some breeding companies produce aneuploid cultivars for commercial reason, i.e. trisomic grape (Park et al., 1999), most aneuploids demonstrate aberrant phenotypes, and their occurrence in crop cultivation can cause serious economic losses.

Aneuploidy is a common problem in the *Brassica* crops and occurs in both pure lines as well as in open-pollinated and F1 hybrid varieties, with rates of aneuploidy exceeding 5% in some lines (Chable et al. 2008, 2009). Aneuploid cauliflowers are recognized by their aberrant phenotypes, and different trisomics give rise to their own characteristic plant- and curd morphology. The specific aberrant morphology is heritable, but their segregation ratios do not usually follow simple Mendelian segregation ratios (Chable et al., 2009). Interestingly, different environmental conditions were suggested to influence the rate of abnormal plant shapes (Wellington 1955), but solid experimental support still lacks. More recently, global changes in DNA methylation were proposed as the cause for the aberrant *Brassica* phenotypes (Chable et al., 2009). Chable and his colleges applied global methylation analysis on cauliflower, however, and their result indicated no signification difference on DNA methylation ratio between aneuploidy and normal plant which could not confirm the hypothesis. We believe the cause of aneuploidy in *Brassica* offspring to lie in unbalanced segregation of chromosomes during meiosis, the details of which will be described in Chapter 4 of this

thesis. Here we will focus on the karyotype analysis of aneuploid based on chromosome painting using repetitive DNA sequences as probes.

Materials and Methods

Plant material

Cauliflower (*B. oleracea* L. var. *botrytis*) lines and their derived diploid and aneuploid progenies were obtained from Rijk Zwaan R&D Fijnaart, the Netherlands.

Repetitive DNA sequence probes

Five repetitive DNA sequences were selected and used for chromosome identification: 1) the 45S rDNA was isolated from the pTa71 plasmid (Gerlach and Bedbrook 1979) using the High plasmid purification kit (Roche, REF: 11754785001); 2) the 5S rDNA plasmid (pCT 4.2, see Campell et al. 1992) was amplified by PCR reaction with the following primers: 5'-GATCCCATCAGAACTTC-3' (forward) and 5'-GGTGCTTTAGTGCTGGTAT-3'(reverse) (Koo et al. 2002); 3) CentBr1 and 4) CentBr2 are centromere repetitive sequences of *B. rapa*, and are known to paint the centromere region of *B. oleracea* (Lim et al. 2007; Xiong and Pires 2011). CentBr1 and CentBr2 both consist of 176 bp repeat motifs (Lim et al. 2005) and are present in different *Brassica rapa* BACs: KBrH001P13 and KBrH015B20 respectively. The two classes repeats share around 82% of their sequences, while within both classes the repeats share over 90% sequence similarity. These centromere-specific repeats were amplified with the primer sets given below, as described by Xiong and Pires (2011):
CentBr1:
forward primer 5'-GAATAGCACAGCTTCATCGTCGTTCC-3'
reverse primer 5'-CTGGGAAACTGTAATCACCTGATCTGAAA-3'
CentBr2:
forward primer 5'-GGGAATATGACACCTTCTTTGTCATTCT-3'
reverse primer 5'-CAGGAAAACTGGGATCACCTGATTTAAAT-3'
5) Lastly, we used a BAC (KBrH092N2) from the *B. rapa* Chiifu 401 genotype library (Parkin et al. 2005), which contains repetitive sequences that paint several chromosome pairs (Xiong and Pires, 2011).

Slide preparation

Young flower buds of cauliflower were fixed in freshly prepared Carnoy solution (pure ethanol: glacial acetic acid, 3:1) and stored in ethanol 70%. Anthers were dissected from the flow-

er buds, washed in Milli-Q and in 10 mM Na-citrate buffer (pH 4.5), and then mildly digested in a pectolytic enzyme mix, of which the stock solution contains 1% cellulase RS (Yakult Pharmaceutical IND.CO, LTD, Tokyo, Japan, Yakult 203033), 1% pectolyase Y23 (pectolyase from *Aspergillus japonicus*, Sigma Aldrich, St. Louis, MO, USA, P-3026) and 1% cytohelicase (cytohelicase from *Helix pomatia*, Sigma Aldrich, St. Louis, MO, USA, C8274). This stock was diluted to a 0.3 % final concentration in Na-citrate buffer, and flower buds macerated for 3 hours at 37 ˚C. After removing the enzyme solution by two washing steps in Milli-Q water, one fragile anther was carefully transferred to a clean slide and dissected with fine needles to make a cell suspension in 15 µL Milli-Q water. Cells were spread on the slide with 25 µL 50% acetic acid for 4 minutes on a 50˚C hot plate. Then 50 µL Carnoy of fixative was applied to precipitate and dehydrate the cells, after which slides were left to air dry for few minutes. We selected only the best slides with well-spread cells, showing chromosomes without cytoplasm for fluorescent in situ hybridization using a phase contrast microscope equipped with 40x or 64x no-cover glass optics.

Fluorescent in situ hybridization

BAC DNA and selected repetitive sequences were labeled with Cy3.5-dCTP (GE Healthcare Life Science, Amersham, UK, REF: PA53521), Cy3-dUTP (ENZO, REF: ENZ-42501), DEAC-5-dUTP (Perkin Elmer Life Sciences, Boston, MA, REF: NEL-455001EA) and simultaneously with Anti-Digoxigenin-Fluorescein (Roche Applied Science, REF: 11207741910) and Rabbit-anti-fluorescein (FITC) (Jackson (bio-connect), REF: 313-096-003) or biotinylated-anti-streptavidin (Vector Lab, CA, REF: BA-0500) and streptavidin, Alexa Fluor 647 (Cy5) (Invitrogen, S21374) with Dig-Nick-translation (Roche Applied Science, REF: 11725816910) or Biotin-Nick-translation (Roche Applied Science, REF: 11745824910, 11725816910)). The BAC probe together with probes of other repetitive sequences were hybridized to various slides of the same plant. Cell spreads were pretreated with 1% formaldehyde for extra fixation (10 minutes at 20 °C), followed by an RNAase treatment (100 µg/mL DNase-free ribonuclease A stock solution, AppliChem, St. Louis, MO, USA, diluted as 1:100 in 2×SSC (Saline-sodium citrate buffer, pH 7)) at 37 ˚C for 1 hour, and then washed again in 2×SSC for 3×5 minutes. Then slides were fixed in 1% formaldehyde at room temperature for 10 minutes. After fixation, slides were washed with 2×SSC and dehydrated in an ethanol series (70%, 90%, and 100% three minutes each).

The DNA probes (10 µL) were added to 10 µl hybridization mixture containing 50% formamide, 20% dextran sulfate, followed by denaturation in boiling water for 10 minutes and put on ice before being added onto the slides. To each slide we applied 20 µL probe mixture, which then was transferred to a 80 ˚C hot plate for a 3 minutes denaturation (to denature chromosomes spread on the slide). This was followed by overnight hybridization in a humid chamber at 37 ˚C. After hybridization, slides were washed at 42 ˚C in 50% formamide/2×SSC for 3×5 minutes, followed by 3×5 minutes washes in 2×SSC. For the de-

tection step we amplified the probe with 500 µg/mL biotinylated-anti-streptavidin (Cy-5) (Vector laboratories, BA-0500, stock solution 1:200 diluted in TNB buffer (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent, Roche Applied Science, REF: 11096176001) or 200 µg/mL Anti-digoxigenin-fluorescein (FITC) (Roche, 11207741910) a stock solution diluted as 1:200 in TNB buffer) and signal amplification (Cy-5) (200 µg/mL streptavidin, Alexa Fluor 647 (Invitrogen, S21374) a stock solution diluted as 1:800 in TNB buffer or 200 µg/mL Rabbit-anti-sheep-fluorescein (FITC) ((Jackson, Bio-connect; 313-096-003) a stock solution diluted as 1:800 in TNB buffer). After the detection steps the slides were dehydrated through an ethanol series (70%, 90%, and 100%, three minutes each). The air-dried slides were counterstained with 12 µL DAPI (4', 6-diamidino-2-phenylindole, Sigma Aldrich, St. Louis, MO, USA, D-1388, dissolved in Milli-Q water, stock solution 100 µg/mL, then dilute

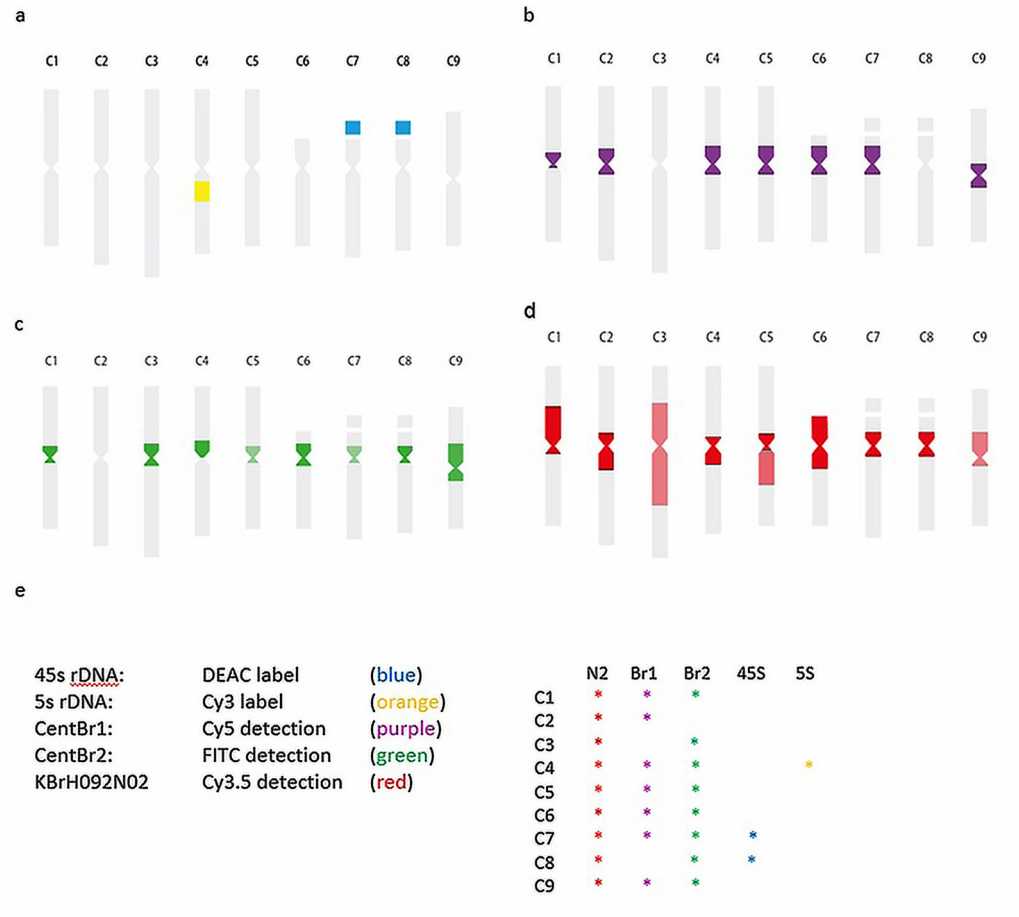


Figure 1. Chromosome karyotype with repetitive sequences: (a) (b) (c) (d) position of different probes on chromosome sets, (e) colors of the respective repetitive probes. The chromosomes identification was based a combinatorial labeling scheme.

as 1:20 in Vectashield (50 µL/mL)), and covered with a 24×50-glass cover slip. The cells were examined with Zeiss Axioplan 2 imaging photomicroscope, equipped with epi-fluorescence illumination and filter sets for DAPI, FITC, Cy3, Cy5, DEAC, and Cy3.5. The images were processed with Genus Image Analysis Workstation software (Applied Imaging), and the selected images were captured by a Photometrics Sensys monochrome 1305 3 1024-pixel CCD camera. Different fluorescent signals were captured and combined by using the multicolour channel mode of Genus software. If needed, we further improved brightness and contrast with Adobe Photoshop CS 6.

Results

Localization of different repetitive sequences on *B. oleracea*

We hybridized all repetitive sequences on the cauliflower slides in a single experiment. Figure 1 displays the painting scheme of used probes. The 45S rDNA (blue DEAC fluorescence), 5S rDNA (orange Cy3), CentBr1 (far-red Cy5), CentBr2 (green FITC) and the KBrH092NO2 BAC repeat (red Cy 3.5). Two 45S rDNA loci are present, located on the distal ends of the short arms of chromosome 7 and 8. Chromosome 4 has a locus of 5S rDNA on the long arm. CentBr1 signals were observed on the centromeres and pericentromeres of chromosomes 1, 2, 4, 5, 6, 7 and 9. CentBr2 has more loci than CentBr2, and is also located on the chromosomes 3 and 8, but lacks on chromosome 2. These four classes of repetitive sequences together distinguish five pairs of the *B. oleracea* chromosomes.

Xiong and Pires (2011) described two *B. rapa* BACs (KBrH092N02 and KBrB072L17) that should allow identification of the remaining two chromosomes. In our hands only KBrH092N02 gave fluorescent signals, which were present on the pericentromeres of all *B. oleracea* chromosomes. This suggests that indeed this BAC contains one or more pericentromere-specific repeats. Since the fluorescent signals of this BAC differ in size and paint different regions of the nine chromosome sets of *B. oleracea*, the BAC could nevertheless be used as chromosome marker. Together with the above four repeat classes we now created a standard set for the karyotype of *B. oleracea* (Figure 1). While six chromosomes sets (1, 4, 5, 6, 7 and 9) are similar in showing signals of BAC KBrH092N02, CentBr1 and CentBr2, the chromosomes could be distinguished as follows:

Chromosome 1 shows a large region painted by KBrH092N02 on the short arm near the centromere region, which is different from all other chromosomes.

Chromosome 2 is unique in lacking a signal of CentBr2.

Chromosome 3 lacks the signal of CentBr1, which is different from other chromosomes.

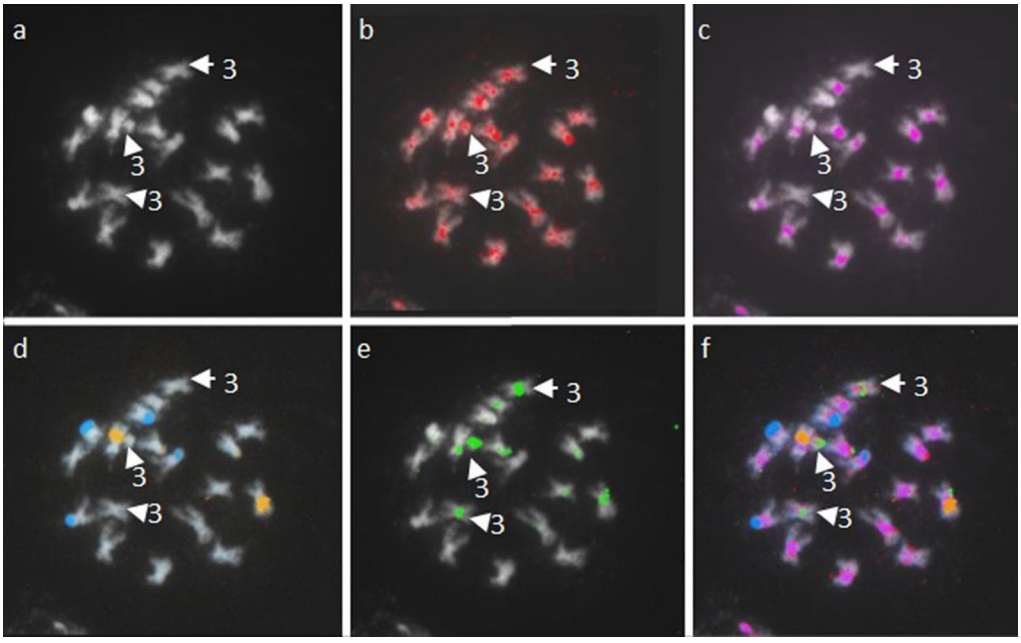
Chromosome 4 is unique in containing 5S rDNA.

Chromosome 5 shows a large region of KBrH092N02 signals on the long arm and small region on the short arm, the short and long arms are clearly distinguished in chromosome 5.

Chromosome 6, KBrHo92No2 paints the centromere region and the entire short arm, which the same painting does not present in other chromosomes.
Chromosome 7 and 8 both contain 45S rDNA (short arm) and N2 (centromere region). However, chromosome 7 has both CentBr1 and CentBr2 foci, while chromosome 8 shows only CentBr2.
Chromosome 9, CentBr2 displays a strong signal compare with other chromosome sets, and does not completely overlap with CentBr1.

Aneuploid identification of cauliflowers with aberrant phenotype

By generating a repeat-based karyotype, we are able to identify the nine pairs of chromosomes of cauliflower and also can identify the aneuploids in this crop. To this end we studied the chromosome complements of various cauliflower plants with aberrant phenotypes (Figures 2, 3 and 4). As several repeats partly overlap in the multicolor FISH, we analyzed the signals in a multi-channel mode of the image analysis software by switching on and off



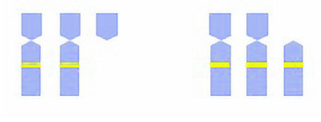


Figure 2. Identification of an unknown aneuploid cauliflower. A five colour FISH to a cell complement shows that the extra chromosome comprises a short arm and centromere region, and not a complete chromosome. This plant is a telotrisomic. (a) DAPI staining of chromosomes, (b) KBrH092N02 (red), (c) CentBr1 (purple), (d) 45S rDNA (blue) and 5s rDNA (yellow), (e) CentBr2 (green), (f) combination of the probes, (g, see below) extra chromosome with only one arm, the yellow band indicated chromosome specific marker for PCR test of trisomy, when the arm with marker loci is missing, it may be seen as disomic.

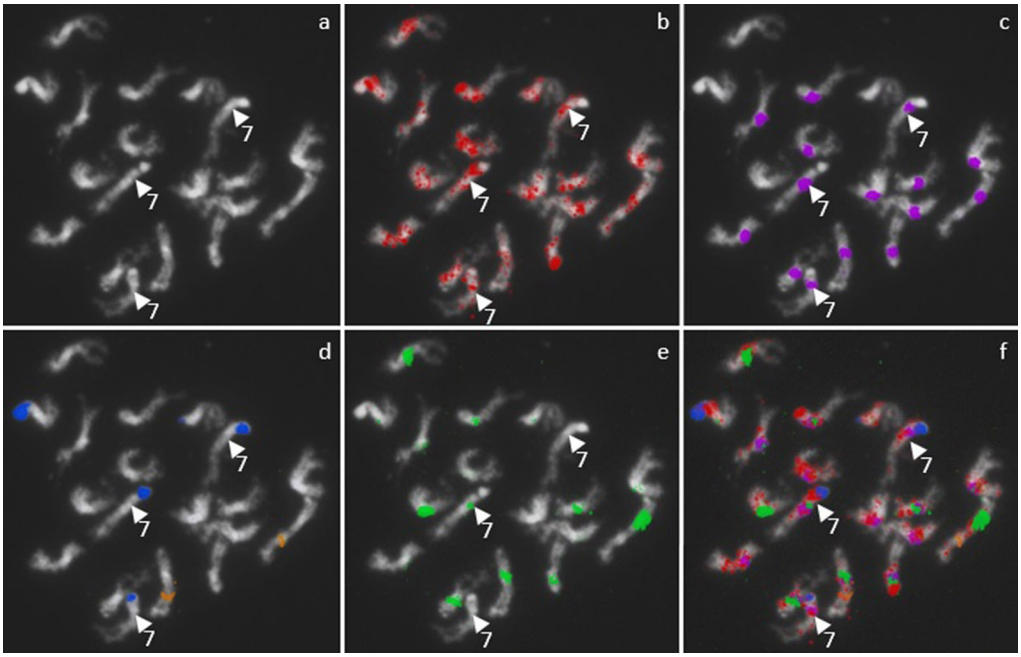


Figure 3. Identification of an unknown aneuploid cauliflower. The FISH results indicate that the extra chromosome is chromosome 7. (a) DAPI staining of chromosomes, (b) KBrH092N02 (red), (c) CentBr1 (purple), (d) 45S rDNA (blue) and 5s rDNA (yellow), (e) CentBr2 (green), (f) combination of the probes.

Plant number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Karyo-type	C9 -S	C7	C4	C6	C8	C7	C3	C6	C3	C2	C4	C5	C5	C8	C4	C8	C6	C7	C1	C2	C6
PCR result	C3	C7	C4	C6	C8	C7	C9	C4	C9	C6	C4	C5	C5	C8	C4	C8	C6	C7	C1	C2	C6
	a,b						a,b	b	b	b											

Table 1. Trisomy identification in different trisomy. a: short arm telotrisomy; b: trisomy identification is different between PCR test and multicolour FISH karyotype analysis.

the signals of the different repeats before combining them together. Among the results obtained we not only observed primary trisomics, of which the extra chromosome was identical to one of the nine chromosome pairs. In addition, we observed plants containing an extra telosome (chromosome arm with centromere) to the cell complement (Figure 2) as well as a tetrasomic or double trisomics of which the two extra chromosomes were from the same or from different chromosome sets (Figures 4a and 4d).

In a control experiment, a sample of twenty aneuploid cauliflowers were identified with a codominant PCR based marker technology that can discriminate between duplex (*AAa*), simplex (*Aaa*) in trisomics and heterozygous (*Aa*) diploids (KASP) (http://www.cerealsdb.uk.net/cerealgenomics/WheatBP/Documents/DOC_SNP_mining.php). In most cases both

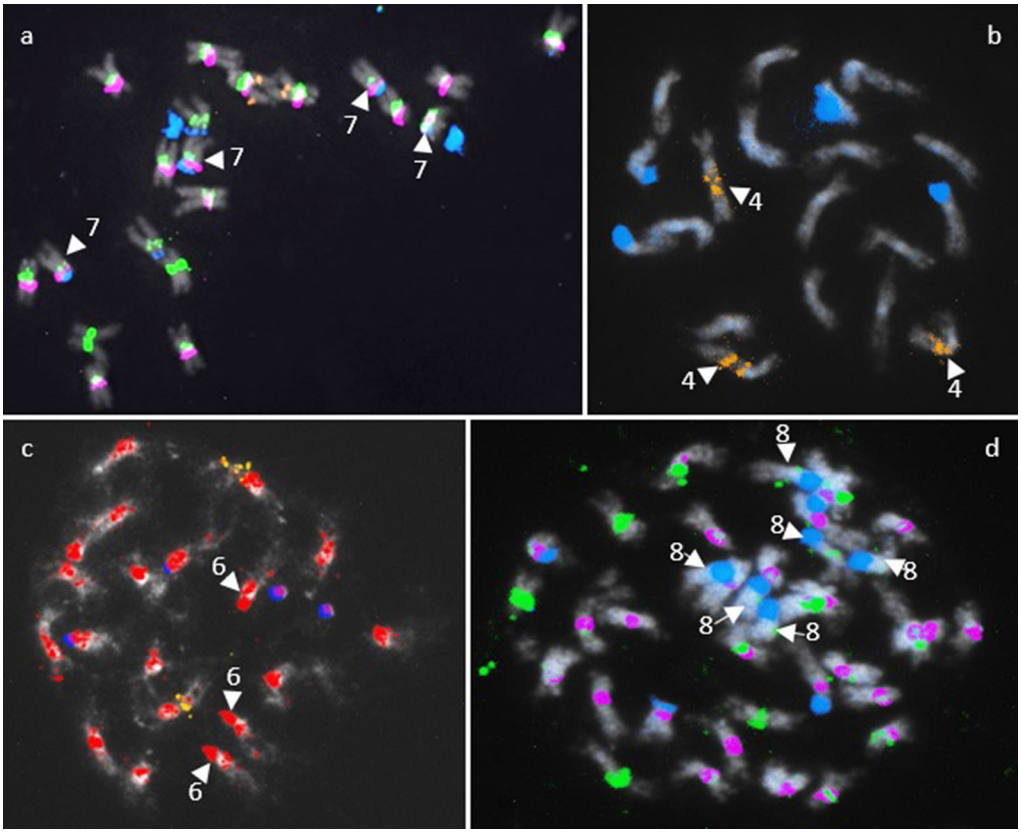


Figure 4. Identification of different unknown aneuploid cauliflower plants, CentBr1 (purple), 45S rDNA, 5s rDNA (yellow) and CentBr2 (green). (a) The FISH result indicates the plant has four chromosomes 7 (a tetrasome for chromosome 7. (b) Primary trisome: the extra chromosome is chromosome 4 (c) Primary trisome: the extra chromosome is chromosome 6 (d) the cauliflower plant is tetraploid, and contains two extra copies of chromosome 8.

methods gave the same results but few discrepancies between the PCR and multicolour chromosome painting were noticed (Table 1). The karyotypes of the plants 1 and 7 were found to contain a telosome, and so could be identified as a telotrisomic. When, as was the case in our control experiment, just one marker per chromosome is used to test for trisomies, aneuploidy in the form of telosomes can go undetected. In this case the marker technology can only distinguish between trisomic and disomic for a given marker. The plants 8, 9 and 10 have also different chromosomes identified by the two methods.

Discussion

Power of the chromosome karyotype for chromosome identification

Repetitive sequences are extensively applied as FISH probe for chromosome identification in plants. Well-known examples are *Arabidopsis thaliana* (Koornneef et al. 2003), maize (Kato et al., 2004; Sadder and Weber, 2001), *Pinus* species (Hizume et al., 2002), Norway spruce (Vischi et al., 2003) and wheat (Mukai et al. 1993). With this method, it is possible to reveal unique repeat banding patterns in chromosomes that without those patterns appear morphologically similar. The technology can also allow to distinguish between different homeologous genomes, alien chromosomes from related species and introgressed regions, like in the hexaploid bread wheat and triticale (wheat x rye hybrids), discriminating the A, B, D and R genomes by their unique repeat banding (Tsujimoto et al. 1997; Fradkin et al., 2013). Also in alien addition lines (from a wild relative of sugar beet) of sugar beet the repeat banding allows identification of the alien chromosomes (Gao et al. 2000). The method was also applied to demonstrating structural and numerical chromosome aberrations (Hizume et al. 2002), as well as to investigate genome evolution by studying replication and diversification of repetitive sequences on plant chromosomes, as sugar beet (Menzel et al. 2008). An important basis for mapping repetitive sequences on chromosomes could be formed by this method which allows the integration between the genetic, molecular, and cytological maps (Chen et al. 2000, Xiong and Pires 2011).

Chromosome karyotyping is the most informative method for establishing numerical chromosome aberrations, and is very precise in determining the type of karyotype aberrations. The technique, though, is laborious and time consuming and hence not suitable for high throughput analysis. As to the molecular marker technology, false negatives may occur in the case of telotrisomics, if just one marker per chromosome is used, and this marker is located on the disomic chromosome arm (Figure 2g). Telotrisomics originate from univalents that break at their centromere during first meiotic division (Friebe et al. 2005, Koornneef and van der Veen 1983). We cannot explain all the differences between the marker-assay and the FISH experiments (Table 1). The occurrence of telotrisomes can be missed by a marker assay when just one marker per chromosome is used, and the marker is disomic (figure 2f). The other discrepancies are more difficult to explain. One explanation might be that some of the used markers were placed on the wrong chromosomes, or that mitotic instability in the plants lead to mosaicism of cell lines with different chromosome sets (*e.g.*, Deng et al. 2010).

Nature of the LTR gypsy elements

Centromere regions of most plants, which include the functional centromere and the large pericentromere, are composed of long arrays of tandem repeats and gypsy type LTR retrotransposons (Sharma et al., 2013). These sequences are evolved rapidly by point mutation, deletion, insertion or mixing of different parental genome sequences during hybridization (Mach 2012, Melters et al. 2013). The centromere classes of *Brassica rapa* and *B. oleracea* are therefore already different, although this differentiation is quantitative rather than quali-

tative (Lim et al. 2007). For our cauliflower material, CentBr1 was present on most chromosomes, including chromosomes 4 and 9, and CentBr2 on most chromosomes, including chromosome 7. Whereas these signals were absent from the mentioned chromosmes in the *B. napus* doubled haploid line TO1000 (Xiong and Pires, 2011). Such repeat differences between related *B. oleracea* varieties might be caused by recent evolutionary changes in part(s) of the centromere repeats. The same may also be true for the unknown repeat in *B. rapa* BAC KBrH092N02 which hybridizes to centromere and pericentromere regions of all chromosomes in our studied material of *B. oleracea*, but we have not tested this repeat on other varieties.

Different cytogenetic karyotype of *B. oleracea*

With two classes of *Brassica* centromere repeats, 45S rDNA, 5S rDNA and repetitive sequences of BAC KBrH092N02, we could identify the nine chromosomes of *B. oleracea*. Repeat polymorphism for the rDNA repeats is obvious if one compares our data with that of Howell (2002), who showed three pairs of 45S rDNA foci on the *B. oleracea* chromosomes 2, 4 and 7, and one 45S rDNA focus on the chromosome that also contains the 5S rDNA. Lim (2007) described there were two chromosomes containing 45S loci in *B. oleracea*, one which co-localized with CentBr1 and CentBr2 and the other co-localized only with CentBr2. It is different from our results in cauliflower where also two chromosomes contain 45s loci. One co-localizes with CentBr1 and CentBr2 and the other co-localized only with CentBr1. Xiong and Pires (2011) demonstrated that chromosomes 4 and 9 of *B. oleracea* do not have CentBr1 loci and chromosome 7 has no CentBr2 locus. In addition, they show that *B. rapa* BAC KBrB072L17 has several loci on *B. oleracea* while B. rapa BAC KBrH092N02 has one locus on only one *B. oleracea* chromosome pair. Although our chromosome study uses the same probe set as Xiong and Pires (2011), KBrB072L17 gives no foci on *B. oleracea*, while KBrH092N02 hybridized on all *B. oleracea* chromosomes.

Future perspectives

Our study clearly demonstrated the power of karyotype analysis in cauliflower based on FISH painting with five different repeat classes. The method not only can identify extra copies of the nine chromosomes, but is also capable of demonstrating additional telosomes in the cell complements. The method that we describe here does have a few drawbacks. Firstly, the method is time consuming and one set of microscopic slides requires a full week for the complete slide preparation, FISH and microscopic analysis. Secondly, literature suggests the repeats that we used are likely polymorphic between different *B. oleracea* varieties and genotypes, and so the use of these repeats has to be tested for any repeat polymorphism in new material.

Beside trisomy identification, chromosome karyotyping is also a nice method to help understanding intraspecific variation in *B. oleracea*, as repetitive sequences are polymorphic between different varieties (Armstrong et al. 1998, Howell et al.2002, Xiong and Pires 2011). Furthermore, chromosome karyotyping describes the different parental genomes in polyploid species (Mukai et al. 1993, Danilova et al. 2014), or can be used to determine homeologous chromosome pairing in interspecific hybrids (Miller et al. 1996, Hao et al. 2011, Nicolas et al. 2007).

Considering the laborious nature of FISH-based karyotyping, the use of marker assays can help in the identification of trisomies. Based on our results, it would be advisable to develop such a marker set with the aid of FISH-based karyotyping, and to develop markers on both chromosome arms. This would allow for the faithful identification of trisomics as well as telosomics in *B. oleracea*.

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Chapter 3

Crossspecies chromosome painting with *Arabidopsis* BACs on cauliflower (*Brassica oleracea* L. var. *botrytis*) for karyotype analysis and chromosome identification

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Abstract

Crossspecies chromosome painting is an advanced Fluorescent *in situ* Hybridization (FISH) technology that is used for hybridizing specific DNA probes of a certain species on the cell complements of a related species under adapted stringency conditions to identify homoeologous sequences. The probe DNA can be isolated from whole chromosomes, or can be obtained from pools of BACs containing DNA sequences of a certain chromosome region. Here we apply this technology by using four pools of BACs from *Arabidopsis thaliana* in a multicolour FISH for banding pattern on the chromosomes of cauliflower (*Brassica oleracea* L. var. *botrytis*. Due to the genome triplication and various chromosome rearrangements of *Brassica oleracea* compared to *Arabidopsis thaliana* we used MUMmer whole genomes alignment plot information to select *Arabidopsis* BAC pools with which all cauliflower chromosomes can be identified. In this paper we show how a set of four BAC pools from the *Arabidopsis* chromosomes 1, 2 and 3, together with 45S rDNA can distinguish all cauliflower chromosomes in the cell complement. We also discuss the power of this tool for identification of aneuploids.

Introduction

Chromosome painting is a Fluorescent *in situ* Hybridisation (FISH) technology that uses chromosomal DNA as a probe for hybridization on the target sequences of a microscopic slide containing spread chromosome complements. In animal systems probe DNAs are generally obtained from microdissected or flowsorted chromosomes and hybridized to the chromosomal target in the presence of unlabelled highly repetitive (Cot1) DNA. In plants such an approach does not work as plant chromosomes are rich in repeats that are dispersed throughout the genome that would paint a greater part of most chromosomes. As a consequence an alternative painting strategy was developed in which probes for chromosome painting are composed of repeatpoor sequences, mostly from selected BACs, YACs, cosmids and other vectors (Schubert et al. 2001). Chromosome painting is not only helpful for chromosome identification and karyotype analyses; it also helps to establish numerical and structural chromosome aberrations. In human and other mammals chromosome painting has been pivotal in diagnosing chromosomebased syndromes (Ried et al. 1998; Speicher and Carter, 2005), characterization of various cancer types (Bishop 2010; Deakin et al., 2012; Kytölä et al. 2000), studying genomic rearrangements (Wienberg and Stanyon, 1997; Müller et al. 1999; Bourque et al. 2004), establishing chromosome specific behaviour during mitosis and meiosis (McKee 2004; Scherthan et al. 1992) and comparing genetic (linkage) maps and physical positions on the chromosomes (e.g., Szinay et al. 2008; 2012).

The first painting technology was called Chromosome *in situ* Suppression hybridization (CISS) where probe DNA is microdissected from chromosome spread preparations, or flow sorted from large number of metaphase chromosomes, then amplified and labelled with De-

generate oligonucleotideprimed (DOP)PCR (Telenius et al. 1992) or Long and Accurate (LA) PCR (Johnson, 1990). This strategy was applied to plant chromosomes to no avail, as plant genomes contain much more dispersed repetitive sequences (Schmidt and Heslop-Harrison, 1998) compared with animal genomes, making it unsuitable for efficient blocking. Besides the abundant occurrence of repetitive sequences, plant genomes also have very similar AT: CG ratio on different chromosomes compared to that of animal genomes, yet again a presumed consequence of repeat homogenization along the chromosomes, that complicates applying CISS painting (Schwarzacher et al. 1997).

As an alternative to CISS hybridization, plant cytogeneticists developed a painting technology in which repeatpoor probes were used that came from isolated vector DNA. Fuchs et al. (1996) used yeast artificial chromosomes (YACs), which contained large chromosome contig inserts as probe for hybridization on tomato and potato chromosomes. Later, contigs or supercontigs of bacterial artificial chromosomes (BAC) clones became much more popular as probes for FISH and were successfully applied in rice and tomato species (Jiang et al. 1995; Dong et al. 2000). Lysak et al. (2001) applied chromosome painting extensively on all five *Arabidopsis* chromosomes with contigs of adjacent BAC clones, and thus made *Arabidopsis* the first plant model that was karyotyped with chromosomespecific BAC contigs.

Later, Lysak et al. (2003, 2006, 2007, 2010; Mandáková and Lysak, 2008) applied the socalled crossspecies chromosome painting (CCP) with probes of *Arabidopsis* BAC contigs on *Brassicaceae* species under adapted stringency conditions. This painting strategy was developed as comparative chromosome painting, and was also successfully applied on potato, tomato and other *Solanaceae* species (Tang et al. 2008; Szinay et al. 2012), sorghum and maize (Amarillo and Bass 2007). The CCP studies from Lysak (2005, 2007) and Ziolkowski (2006) revealed a unique evolutionary genome triplication, *i.e.* as a result of which single *Arabidopsis* contigs always revealed three copies on two or three pairs of chromosomes in diploid *Brassica* species, including *Brassica rapa*, *B. nigra* and B. *oleracea* (Lagercrantz et al. 1996a,b; Lysak et al. 2005, 2007; Ziolkowski et al. 2006). To further elaborate on this genome triplication Kaczmarek et al. (2009) compared the genomes of *A. thaliana* and *B. oleracea* with their genetic linkage maps, and showed that three or four copies of *Arabidopsis* probes were represented in the *Brassica* genome. Comparable analysis of Parkin et al. (2005) demonstrated four to seven copies of *A. thaliana* in the allotetraploid *B. napus*. Wang et al. (2011) who presented the draft genome sequence of Chinese cabbage (*B. rapa*) established segmental collinearity of the genomes of *B. rapa* and *A. thaliana*, thus confirming the almost complete triplication of the *B. rapa* genome relative to *A. thaliana*.

With the triplication of *B. rapa* in mind, and the assumption that *B. oleracea* demonstrates a comparable genome structure, we hypothesized that crossspecies chromosome painting with probes from *Arabidopsis* BACs would display three pairs of fluorescent signals on the cauliflower chromosome complement. By selecting specific combinations of pooled *Arabidopsis* BACs representing contig blocks with known genomic positions on the *B. oleracea* chromosomes, we expected to create unique multicolour FISH patterns, where

all individual *Brassica* chromosomes could be identified. In this study, we describe the first construction of such a multicolour crossspecies FISH labelling for the identification of cauliflower (*B. oleracea* L. var. *botrytis*) chromosomes.

Material and Method

Plant materials

B. oleracea L. var. *botrytis* genotypes and derived diploid and aneuploid progeny plants were obtained from Rijk Zwaan R&D Fijnaart, the Netherlands. The *Brassica* that we used for genome comparison came from the sequenced "walking stick kale" (*B. oleracea* var. *longata*) from Tenerife, Canary Islands, which should not be confused with the Jersey kale from the Isle of Jersey.

Sequence comparison

B. oleracea var. *longata* was sequenced in collaboration with Dr. Frederic Lens (Naturalis Biodiversity Centre, Leiden, the Netherlands). The reference assembly was done by Patrick Edger at University of Missouri using the "TO1000" genome as reference (http://brassica.jcvi.org/cgibin/brassica/index.cgi). The B. *rapa* genome v1.0 was obtained from the NCBI FTP site, while the *A. thaliana* genome v10 was obtained from the TAIR database. The relation between the three genomes was studied using the Maximum Unique Matcher MUMmer v3.23 (Kurtz et al., 2004). This software exports only the best alignments ordered in such a way that they create the longest diagonal assuming a one-to-one relationship between the sequences. After that, the result is filtered to leave only the longest consistent alignment between the genomes, permitting rearrangements and excluding alignments smaller than 5 Kbp or alignments with sequence identity or sequence uniqueness smaller than 10%.

BAC pool1: Arabidopsis chromosome 1	T23E18	F15M4	F14G6	F28O16	F7O12	F22K20	T14N15	F2P24	T5M16	T32E8	F28K19
BAC pool2: Arabidopsis chromosome 1	T23J18	F25C20	F12F1	T28K15	T12C24	F13K23	F3F19	T6J4	F13B4		
BAC pool3: Arabidopsis chromosome 2	F3G5	F13M22	T8P21	F16M14	T19C21	T6A23	F13I13	T7F6	T16B24	F12L6	
BAC pool4: Arabidopsis chromosome 3	T21P5	T12J13	F20H23	T11I18	T6K12	T27C4	F7O18	T9J14	T12H1	F22F7	

Table 1. *Arabidopsis* BACs from chromosome 1, 2 and 3 for cross-species painting, the BACs contained low repeats (Dr Mandáková, pers. comm.).

BACFISH probes contain *Arabidopsis* BACs

For the design of a painting scheme to identify *B. oleracea* chromosomes using *A. thaliana* BACs, we selected forty *Arabidopsis* BACs from the chromosomes 1, 2 and 3 (Table 1, and Results section). Each BAC pool was labeled in one colour. These BACs were provided by the Arabidopsis Biological Resource Center (Columbus, OH, USA, https://www.arabidopsis.org/). BAC DNA was isolated with high purification kit (Roche, Germany), and amplified with REPLIg kit (Qiagen, Germany).

Repetitive DNA sequence probes

Besides the *Arabidopsis* BACs, we also used 45S rDNA as a landmark for the *B. oleracea* L. var. *botrytis* NOR chromosomes. The 45S rDNA was isolated from the plasmid pTa71 with the High plasmid purification kit (Roche) (Gerlach and Bedbrook 1979).

Slide preparation

Preparation of chromosome spreading slides was described in Chapter 2.

Fluorescent *in situ* Hybridization

The FISH protocol that we followed is essentially that in Chapter 2, with some adaptations. After degrading the RNA with RNase, slides were treated with pepsin (Sigma Aldrich, St. Louis, MO, USA) that was diluted from stock solution of 100 mg/mL in 10 mM HCl in a ratio of 1:100 in 10 mM HCl) to remove cytoplasm covering the chromosomes as much as possible. After dehydration through an ethanol series (70%, 90%, and 96% series) and airdrying, we checked the slides in the phase contrast microscope to see if cytoplasm was sufficiently removed. Probe hybridization on slides was carried out 3648 hours. After hybridization, slides were washed three times at 42 ˚C in 20% formamide/2×SSC to get higher hybridization ratio. Details on fluorescence microscopy, and image capture and processing are described in Chapter 2.

Results

Comparison of *A. thaliana*, *B. oleracea* and *B. rapa*

To develop a proper painting scheme for the identification of all *B. oleracea* chromosomes a number of comparative genome analyses of homeologous segments between the two species had to be carried out. To this end we visualized a whole genome alignment by revealing segmental collinearity using the Maximum Unique Matcher (MUMmer 3.23) software. The

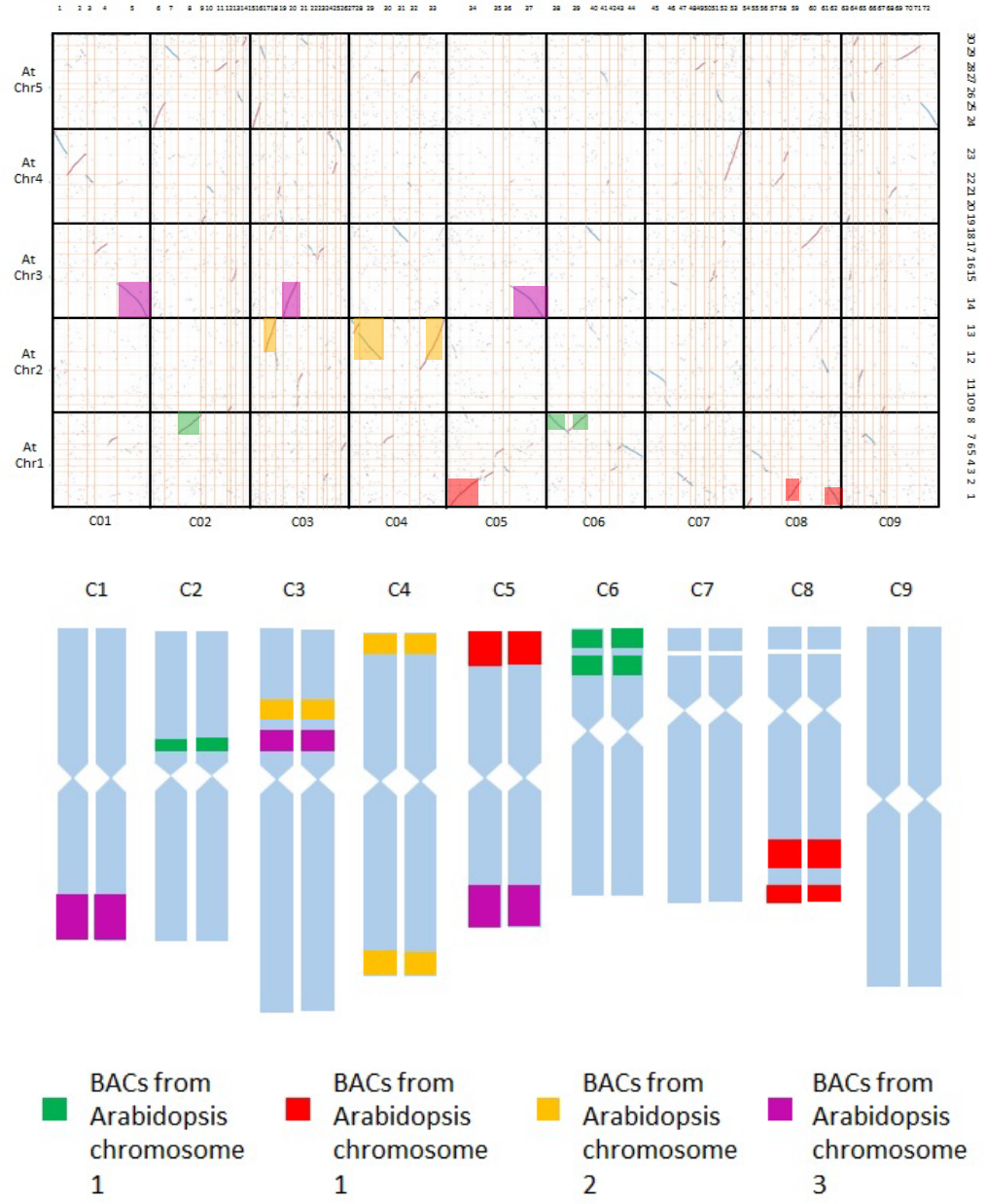

Figure 1. MUMmerplot of the genomes *Arabidopsis thaliana vs. Brassica oleracea*, and a painting scheme for cross-species FISH a) Genome comparison between *A. thaliana* and *B. oleracea*, different color blocks indicate *Arabidopsis* sequences, which were hybridized to *B. oleracea* for chromosome karyotyping. b) Chromosome karyotype of *B. oleracea* with hybridized *Arabidopsis* sequences.

parameter settings were first tested for *B. rapa* (Supplementary Figure 1S) and then compared with the previously published MUMmer plot of the same species (Wang et al. 2011). This showed that our MUMmer plot settings produced the same matches as those in the study of Wang et al. (2011). The plot confirms the same genome triplication, and numerous larger and smaller chromosome rearrangements (inversions and translocations). We then constructed a similar MUMmer plot for the five chromosomes of *A. thaliana vs.* the nine chromosomes of *B. oleracea* (Supplementary Figures 2S), and that for *B. oleracea vs. B. rapa* (Supplementary Figure 3S).

The plot for the genomes of *A. thaliana vs. B. oleracea* showed a highly identical pattern of triplication as that for the genomes *A. thaliana vs. B. rapa*, with only few sequences that are inverted between the two plots. Sequences of *A. thaliana* chromosome 1 aligned with *B. oleracea* chromosome 8, while the same sequences of *A. thaliana* are inverted on the genome of *B. rapa* chromosome 9 (Supplementary Figures 1S and 2S). The plot for *B. oleracea vs. B. rapa* explains the high similarity of Figures 1S and 2S.

The plot of the *B. oleracea* and *B. rapa* genomes demonstrated the expected high similarity between the two homeologous genomes (Supplementary Figure 3S). All *B. oleracea* contigs have at least one copy in the *B. rapa* genome and some contigs have two or three copies. Chromosomes C01, C02 and C03 of *B. oleracea* were nearly completely collinear with chromosome A01, A02 and A03 of *B. rapa*. Contig 15, 16, 17 of *B. oleracea* chromosome 4 were collinear with *B. rapa* chromosome 4. We also found some inverted regions between the two genomes which corresponded to and corroborated the above explained differences that were found when *A. thaliana* was compared with both species.

Selection of BAC pools for crossspecies FISH

Using the MUMmer plot of *A. thaliana* and *B. oleracea* (Supplementary Figure 2S) we selected a number of contigs to paint *B. oleracea* chromosomes. These contigs, derived from *Arabidopsis* chromosomes 1, 2, and 3 are shown in Figure 1, with the resulting color scheme on *B. oleracea*.

We used a list of selected *Arabidopsis* BAC clones that contain relatively low amount of repetitive sequences (Table 1, Dr Mandáková, pers. comm.). Four contigs of a total of 39 BAC clones of *Arabidopsis* chromosome regions where now used, together with 45S rDNA for the NOR chromosomes, which together identifies all nine pairs of *B. oleracea* chromosomes. The BAC sets of each contig were labeled with different colors for chromosomes identification.The complete list of BAC pools used in our experiments in given hereafter.

***Arabidopsis* chromosome 1**: Contig 1 aligned with *B. oleracea* chromosomes 5 and 8, the contig was inverted on chromosome 8. Contig 8 aligned with *B. oleracea* chromosomes 2 and 6 and is in inverted orientation with chromosome 6.

***Arabidopsis* chromosome 2**: Contig 12 and 13 aligned with the *B. oleracea* chromosomes 3 and 4, the two contigs both aligned and were in inverted orientation with chromosome 4

***Arabidopsis* chromosome 3**: Contig 14 aligned with *B. oleracea* chromosome 3, and is in inverted orientation on chromosomes 1 and 5; Contig 18 aligned on *B. oleracea* chromosome 8, and are in inverted orientation with chromosomes 4 and 6.

Karyotype of *Arabidopsis* BAC on *Brassica oleracea*

Using *Arabidopsis* BAC contigs of chromosomes 1, 2 and 3 and 45S rDNA, we could identify nine sets of chromosome of *B. oleracea* (Figure 2). Colours re based on the scheme in figure 1, and chromosomes can be characterized as follows:

Chromosome Co1: Contig 14 of *Arabidopsis* chromosome 3 localizes on the distal end of the long arm.

Chromosome Co2: Contig 8 of *Arabidopsis* chromosome 1 is on the pericentromere region of the short arm.

Chromosome Co3: Contigs 13 and 14 from *Arabidopsis* chromosomes 2 and 3 are next to each other on short arm. Contig 14 localizes between the centromere and Contig 13.

Chromosome Co4: Contig 13 of *Arabidopsis* chromosome 2 localizes on the distal region of both arms.

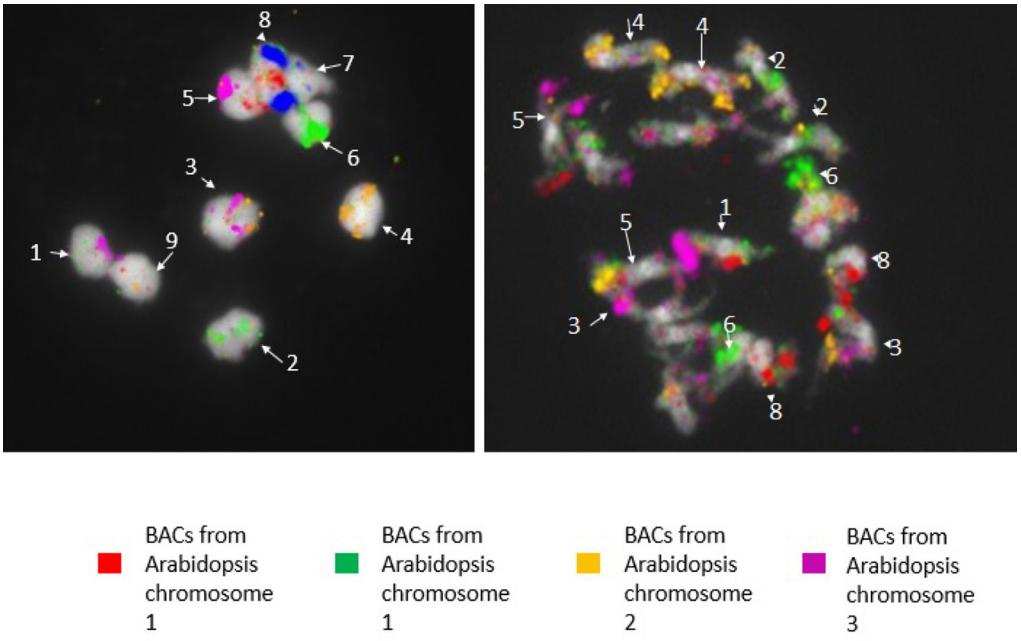

Figure 2. Multicolor chromosome painting using selected pooled BACs of *Arabidopsis* chromosomes 1,2 and 3 (cf. Figure 1) on cauliflower chromosome sets. a) Pollen mother cell at diakinesis of *B. oleracea* in which nine chromosome pairs can be discerned. The probe schemes for the *Arabidopsis* BACs are provided. The blue (DEAC) fluorescence is from the 45S probe showing the NOR chromosomes. b) Multicolor FISH on a *B. oleracea* mitotic metaphase complement. Here only *Arabidopsis* BACs were used, without the 45S rDNA

Chromosome Co5: Contig 1 of *Arabidopsis* chromosome 1 localizes at the end of the short arm, while Contig 14 of *Arabidopsis* chromosome 3 localizes on the end of the long arm.
Chromosome Co6: Contig 8 has two copies that localize next to each other on distal region of the short arm.
Chromosome Co7: 45s rDNA is at the telomere region of the short arm.
Chromosome Co8: 45s rDNA is locates on the distal region of the short arm and contig 8 has two copies next each other on the distal region of the long arm.
Chromosome Co9: no contig or 45s rDNA is localized on this chromosome.

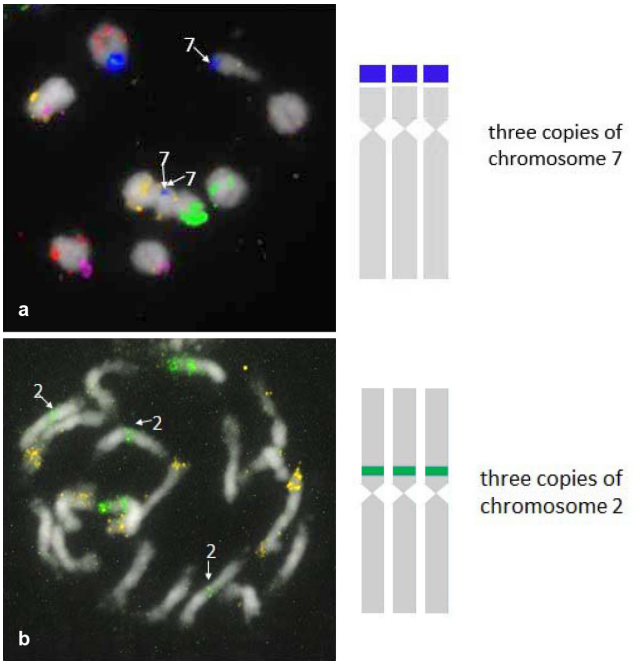

Figure 3. Identification of an unknown aneuploid of *B. oleracea* by cross-species chromosome painting.
a) FISH of pollen mother cell at diakinesis identifies the extra chromosomes as nr. 7. Two chromosomes 7 are paired but the third homologue remains as a univalent. b) BACs from *Arabidopsis* chromosome 1 (green) and chromosome 2 (orange) were used for a cross-species FISH. The FISH identifies the extra chromosome as CO2.

Identification of aneuploids in cauliflower

With crossspecies chromosome painting, we could distinguish all the chromosomes of *B. oleracea* and identify different trisomics among cauliflower offspring (Figures 3a and b). As all four pools of *Arabidopsis* BACs have three copies on the *B. oleracea* genome, we see six foci for each BAC pool on normal cauliflower chromosomes (Figure 2). In the case of a primary trisomy, containing three copies of one of the chromosomes, the crossspecies painting produces seven or eight pairs of foci, if the extra chromosome has two copies of the same contig. Figure 3 shows a diakinesis cell of a plant trisomic for chromosome 7, where the extra chromosome appears as a univalent. In another example a tapetum cell at mitotic prometaphase shows seven green fluorescing foci, identifying the extra chromosome as Co2.

Discussion

This study is the first of its kind where crossspecies painting is used for a plant species with a triplicated genome structure using probe DNA from a model species (*Arabidopsis*). The advantages of this technology are, compared to other chromosome banding and painting strategies, very clear. Firstly, there is an almost endless choice of *Arabidopsis* BAC pools for the identification of individual chromosomes, but such probes are also eminent for the identification of minor chromosome domains, and so are most appropriate for demonstrating large chromosome rearrangements, as was used for the characterization of an inversion in Chapter 5 of this thesis. Secondly, the use of *Arabidopsis* BACs with known DNA sequences directly points to the homeologous region in the *Brassica* genome and so makes it easier to correlate genetic and physical maps. Thirdly, the *Arabidopsis* probe DNA and target *B. oleracea* DNA are different in their repeat content and so this painting method outperforms traditional BAC FISH painting in the sense that repetitive elements do not hybridize to the target genome and do disturb the specific signals of the single copy sequences (cf. the supplementary Figures 1S, 2S and 3S).

Crossspecies chromosome painting is still a young technology and has been applied to a limited number of other plant species, including members of the *Brassicaceae* and *Solanaceae* families (Lysak et al. 2007; Tang et al. 2008; Szinay et al. 2012). The previously developed FISH karyotyping on some *Brassica* species including *B. napus* (Xiong and Pires, 2011), *B. rapa* (Koo et al. 2004), *B. oleracea* (Armstrong et al. 1998) and *B. nigra* (Fukui et al. 1998) used only the 45s rDNA and pericentromere repetitive sequences and do have some limitations as explained by Armstrong et al. (1998), Howell et al. (2002), Xiong and Pires (2011) and in Chapter 2.

Genome duplication in evolutions

Recent molecular biosystematic studies convincingly demonstrated ancient whole genome duplications in most of the plant families (Debodt et al., 2005; Schranz et al. 2012). Genome sequencing indicated that 60% of the *Arabidopsis* sequences duplicated in 24 segments in Brassica (Lysak et al. 2001). Other studies revealed that some genome contigs of *Brassica* species are triplicated compared to the *Arabidopsis* genome (Lysak 2005, Cheng et al, 2014). The MUMmer genome comparison between *A. thaliana, B. rapa* and *B. oleracea* genome sequences clearly indicated that most *B. rapa* and *B. oleracea* genome contigs have three copies from *A. thaliana* genome, and few contigs have one, two or four copies (Supplementary Figures 1S and 2S). The similar alignment patterns suggested that *B. rapa* and *B. oleracea* genomes are have largely colinear contigs (Supplementary Figure 3S). According to the MUMmerplot result, most *B. oleracea* genome contigs have one copy on the *B. rapa* genome, while some have two or three copies. Besides large contigs, the two species also share plenty of repeats. *Brassica* species are also considered to be an ideal model to investigate

the evolution of polyploids, as was already known since the work of Nagaharu (1935) who showed that *Brassica* allopolyploids were formed from the three diploid ancestors: *B. rapa* (A genome), *B. nigra* (B genome) and *B. oleracea* (C genome) (Snowdon, 2007). Of the three diploid genomes *B. oleracea* and *B. rapa* are more closely related, while the *Brassica nigra* genome is different from the other two genomes. Liu et al. 2014 made a genome comparison between *A. thaliana, B. rapa* and *B. oleracea*, showing numerous chromosome rearrangements, asymmetrical gene loss and the asymmetrical amplification of transposable elements between the three genomes, suggesting a differential evolution of these genomes. Paritosh et al. (2014) performed a synteny analysis between the A and B genomes in the genome of *B. juncea* (AABB), the tetraploid containing genomes from *B. nigra* (BB) and *B. rapa* (AA). The two genomes showed a striking diversification in the arrangement of gene blocks and reported genome fragmentation patterns: patterns that could not be explained simply by assuming inversions or translocations, and so they proposed the two genomes evolved from independent hexaploidization events, after which they underwent genome reduction.

Cross-species painting

Chromosomespecific BAC FISH painting in plant is often complex because large vector DNAs may contain too many repetitive sequences. Even if probe DNA is derived from a related species, repetitive sequences may obscure a clear FISH pattern of the single copy sequences as repetitive elements in the probe DNA hybridize throughout the target genome. It is therefore beneficial to compare the genomes of the two species to which cross species FISH is to be applied. When the genomes of the related species are highly similar, there is a high likeliness of the crossspecies FISH being affected by repeat elements. Cheung et al. (2009) compared BACs from *B. oleracea* and *B. rapa*, and concluded a close similarity between their sequences. A similar impression is obvious if one compares the MUMmer plots from the same species (Supplementary Figure 3S). In other words, using *B. rapa* BACs as probes on *B. oleracea* chromosomes will likely be problematic.

Trisomy identification with *Arabidopsis* BAC FISH

The cross-species chromosome painting that we presented in this study convincingly demonstrated the power for karyotype analysis and chromosome identification in cauliflower. With this technique we are able to establish primary trisomics in the progeny of cauliflower breeding material with high rates of aneuploid offspring. The next step is to work out painting sets using pools of BACs from the five *Arabidopsis* chromosomes to make a detailed multicolour banding pattern covering all cauliflower chromosomes completely. Such painting sets enable us to diagnose fast and precisely not only numerical aberrations, but also large inversions and translocations in and between the cauliflower chromosomes.

We also foresee the need for simplifying the BAC FISH technology to use PCR based amplicons for a fast and convenient source of probe DNA for these painting studies.

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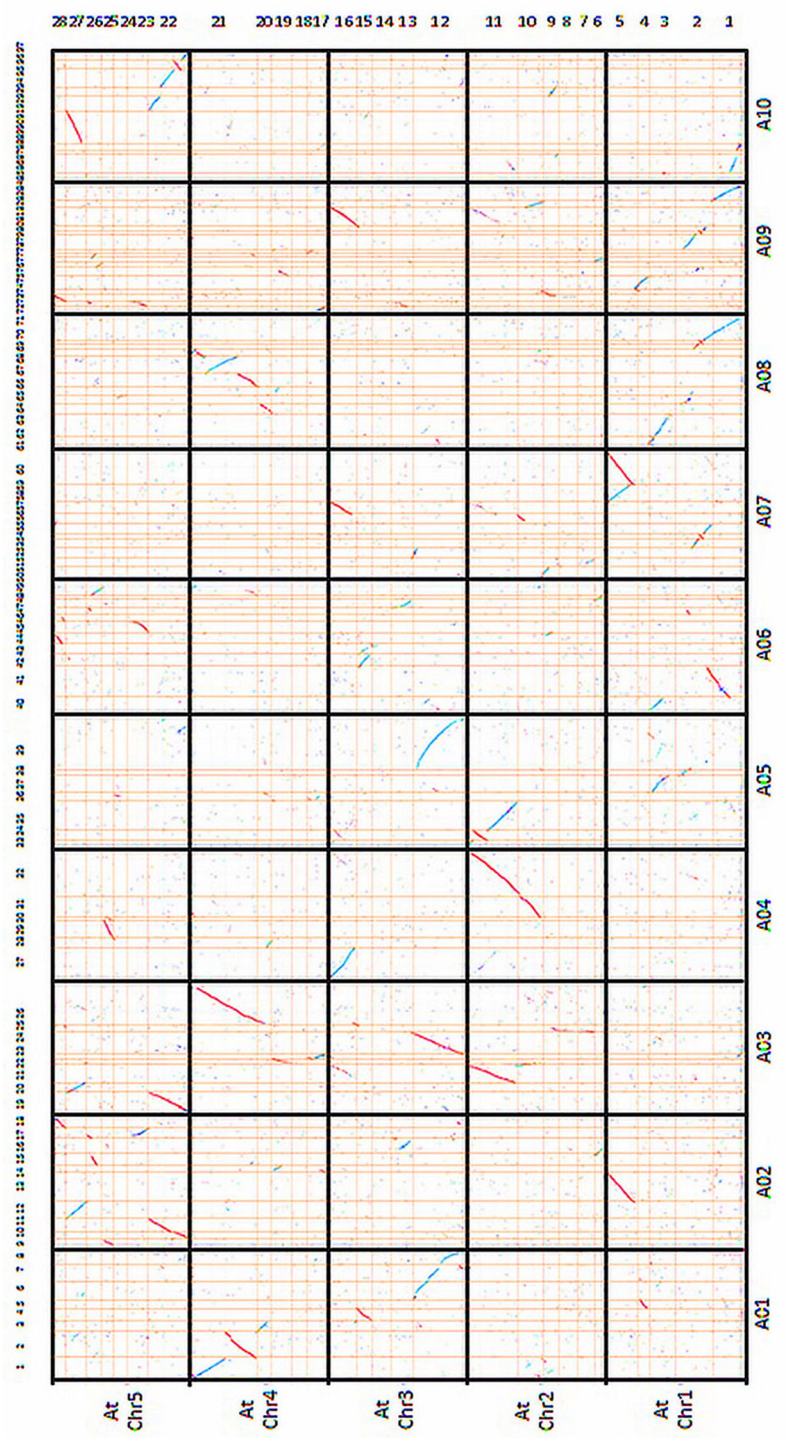
Supplementary data (following pages)

Supplementary Figure S1. MUMmerplot showing the genome relation between *Arabidopsis thaliana* (x=5) and *Brassica rapa* (x=10). The right and top numbers along the axes refer to the synteny blocks of the two genomes. Red lines are collinear; blue lines are inverted regions. Several inversions and translocations are obvious form the figure.

Supplementary Figure S2. MUMmerplot of genomic relations between *Arabidopsis thaliana* (x=5) and Brassica oleracea (x=9). The coding is as in Suppl. Figure 1.

Supplementary Figure S3. MUMmerplot comparison of *Brassica rapa* (x=10), xaxis and *Brassica oleracea* (x=9), yaxis. The smaller dots with accumulations in the pericentromeres are the many dispersed repetitive sequences that the genomes of both species have in common. The plot also displays the perfect collinearity between some of the homeologous chromosomes, e.g., A01/C01, A02/C02, while other homeologues show translocations (e.g., A04+A05/C05) and inversions (A07/C06) that have occurred between the two species.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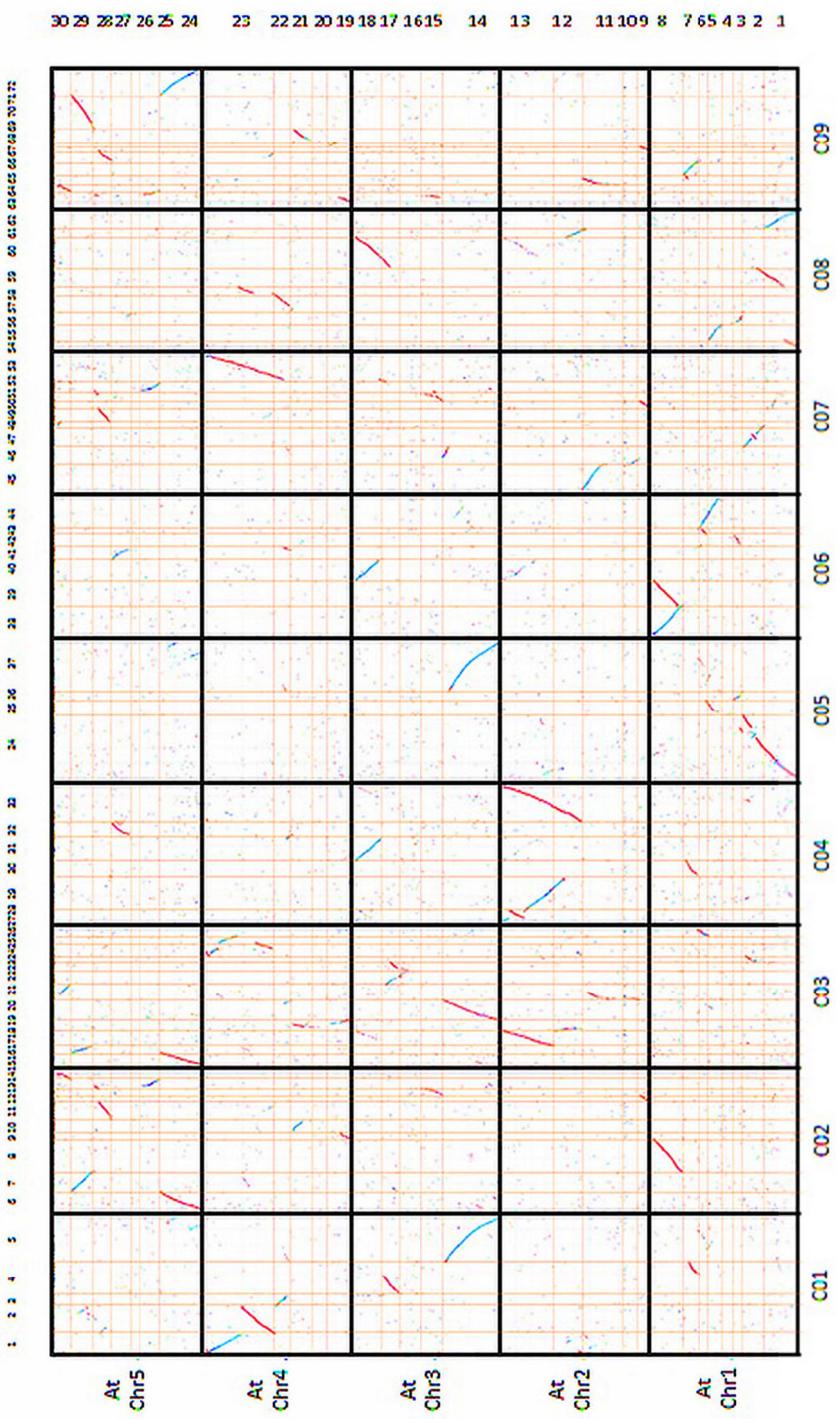
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23
22 21 20 19 18 17 16 15
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C09
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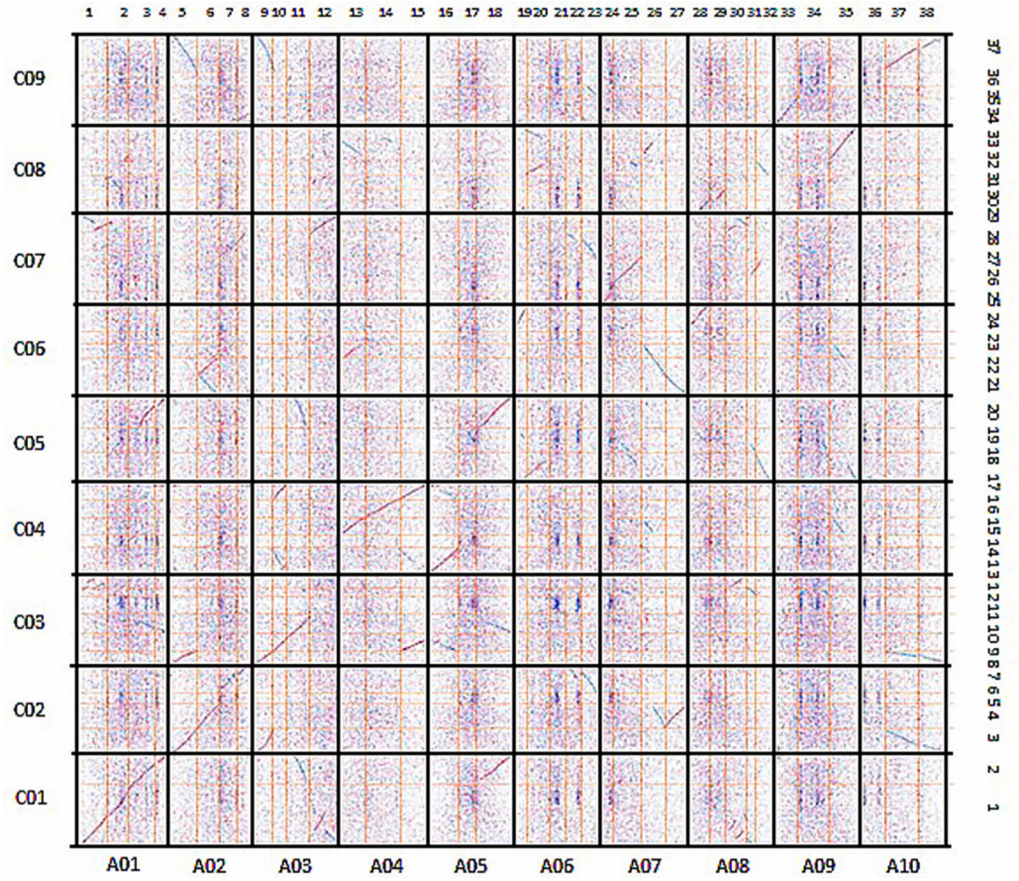
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Chapter 4

Meiotic aberrations leading to aneuploidy in Cauliflower (*Brassica oleracea* L. var. *botrytis*)

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Abstract

The occurrence of trisomics offspring during cauliflower cultivation (*Brassica oleracea* L. var. *botrytis*) is a serious problem since aneuploidy offspring produce aberrant curds. In spite of extensive breeding efforts, the selection of genotypes producing balanced haploid gametes is problematic as both genetic and environmental factors influence the prevalence of aneuploid gametes. In this chapter, an analysis of different meiotic stages in pollen mother cells of cauliflower is presented to reveal the origin of unbalanced gametes. To this end we compared three lines that generate <5%, 5-10% and >10% aberrant plants among the offspring. Spread pollen mother cells show regular pairing between the homologues at pachytene whereas at diakinesis numerous univalent pairs were observed, suggesting that meiosis is desynaptic and that chiasma formation is incomplete or disrupted. Later meiotic stages displayed numerous cells with unbalanced chromosome numbers. Fluorescence immunostaining of MLH1 showed lower numbers of fluorescent foci in the line with highest rate of aberrant offspring, but the number of observations was not enough to obtain certainty on whether crossovers in a specific class are affected. Interchromosomal connections were also found at diakinesis and metaphase I, which were shown, in part, to be composed of centromeric and 45S rDNA tandem repeats. These connections disappear at later stages and seem not to affect the balanced segregation of the half bivalents at anaphase I. The presence of MLH1 foci at the sites of bivalent connections suggests that these originate from DNA repair though homologous recombination. We subsequently investigated whether desynapsis and chromosomal interconnections may be result from the same mutations that cause the development of the typical compact head of the cauliflower phenotype. To this end we studied meiosis in the *APETALA1*/ *CAULIFLOWER* double mutant of *Arabidopsis*, which also exhibits the characteristic curd morphology of cauliflower. Metaphase I in this double mutant *Arabidopsis* did show some bivalent interconnections, but univalents were not observed.

Introduction

Meiosis is a special cell division that precedes gamete formation and sexual reproduction in eukaryotes. A single round of nuclear DNA replication is followed by two consecutive cell divisions. During the first meiotic division, chromosome sets are halved, leading to two haploid daughter cells, which then undergo a second mitotic division in which their chromatids segregate. Meiosis produces new allele combinations through interchromosomal and intrachromosomal (or crossover) recombination of parental alleles. Chromosome recombination follows from the random orientation of chromosomes and chromatids at meiosis I and II, while crossover recombination results from specific processes during meiotic prophase. During meiotic prophase, homologues pair (synapsis) during which crossovers are formed that later become visible as chiasmata, and ensure the balanced disjoin of homo-

logues at metaphase I (reviews in Dawe 1998; Zickler and Kleckner 1999; Cnudde and Gerats 2005; Ma 2006; Harrison et al. 2010).

Synapsis occurs during the meiotic prophase, when homologous chromosomes pair, align and form a synaptonemal complex (SC) (Gillies, 1984). It connects paired homologous chromosomes during meiosis in most species, although some species like fission yeast do not form an SC (Sym et al. 1993, Nag et al. 1995). The SC associates with recombination nodules (RNs), which are the sites at which meiotic double strand breaks are repaired (Abirached-Darmency et al. 1983; Albini and Jones, 1988; Sherman and Stack, 1995; Stack, 1989). At diakinesis, paired homologous chromosomes are referred to as bivalents. When an SC is not formed and/or crossover formation fails, this leads to meiotic aberrations in the late prophase of meiosis; the first group of defects is referred to as asynapsis in which chromosomes (partly) fail to pair or synapse. Examples are mutations in genes involved in the upstream part of meiotic recombination machinery, such as *ASY*1 and *SPO*11 (*e.g.*, Wei and Zhang 2010). The second group is that of desynaptic mutants, which show regular chromosome pairing, or synapsis (i.e., the formation of an SC), but fail to complete crossover formation, resulting in no or less chiasmata (de Muyt et al., 2009). Other meiotic aberrations can also lead to the unbalanced segregation of chromosomes, like merotelic kinetochore attachment in which a kinetochore attaches to microtubles originating from both spindle poles (Shi and King, 2005). Chromosome non-disjunction is another phenomenon in which meiotic or mitotic chromosomes or chromatids do not segregate properly during cell division and hence may result in aneuploidy. In the case of mitotic non-disjunction it generates mosaicism in which aneuploid and normal cell lines arise. When this occurs during the somatic cell divisions during oogenesis or spermatogenesis, it may cause germline mosaicism, and an increased rate of aneuploidy in eggs or sperm (Robinson and McFadden, 2002). Other meiotic disturbances that may give rise to aneuploidy or changes in ploidy are (aspecific) stickiness, premature loss of sister-chromatid cohesion, failure of cytokinesis, microtubule errors, and first and second division restitution (FDR, SDR), and were described regularly in plants like *Arabidopsis thaliana* (Castellano and Sablowski 2008, Yang et al. 1999, Zamariola et al. 2014), *Brassica napus* (Souza and Pagliarini 1996), wheat (Huskins and Hearne, 1933), maize (Beadleg 1933, Caetano-Pereira et al. 1995) and tomato (Soost, 1951). Unbalanced gametes may give rise to aneuploid offspring, as was shown for several plant species such as *Arabidopsis* (Grelon et al. 2003), wheat (Griffiths et al. 2006) and maize (Carlson et al. 2007). Aneuploids with aberrant phenotypes are pretty common in *Brassica* crops. Most striking is the situation in cauliflower where a high incidence of aneuploidy that leads to small and irregularly shaped curds causes considerable economic loss to growers and breeders. The genetic basis for these high rates of aneuploidy is still unclear. Previous studies have shown that the rate of aberrant plants varies depending on the genotype or region of cultivation. Mutant phenotypes can thus reverse to normal phenotypes even though the occurrence of aberrant phenotype has a heritable component (Chable et al. 2008). In a later study, Chable

et al. (2009) suggested an epigenetic explanation, as aneuploidy could not be related to the aberrant phenotype.

In this study, we focus on possible erratic processes during male meiosis that lead to unbalanced gametes and to aneuploid offspring. We suggest that univalent formation by desynapsis is the most obvious explanation for unbalanced chromosome segregation at anaphase I. We also screen meiotic cells for non-disjunction and aspecific connections between the chromosomes. We subsequently ask whether mutations leading to curd formation could, as a side effect, induce erratic meiosis. We therefore study the *APETALA*1/ *CAULIFLOWER* double mutant of *Arabidopsis thaliana* (Bowman et al. 1993, Smyth 1995), which shows the typical curd like morphology as seen in cauliflower. The *APETALA*1 mutation causes flower meristems to partially convert into inflorescence shoots by affecting the outer two whorls that normally develop into sepals and petals. The *CAULIFLOWER* mutation enhances the phenotype of *APETALA*1 mutant greatly (Bowman et al. 1993).

Material and Methods

Plant material

For this cytogenetic analysis of male meiosis we selected three lines of cauliflower (*B. oleracea* L. var. *botrytis*) that differ in the incidence of aneuploidy among their progeny. The so-called **Good Line** produces less than 5% aneuploids in its offspring; the **Moderate Line** produces 5-10% aneuploids, whereas the **Bad Line** produces more than 10% of aneuploids. The *APETALA*1/ *CAULIFLOWER* double mutant of *Arabidopsis thaliana* was obtained from Dr. Kerstin Kaufmann (Bioscience, Plant Research International, WUR, Wageningen (Bowman et al. 1993).

Selection of repetitive DNA sequence

Three repetitive DNA sequences were selected for chromosome identification of *B. oleracea* L. var. *botrytis*: 45S rDNA, CentBr1 and CentBr2. The 45S rDNA (plasmid pTa71, see Gerlach and Bedbrook 1979) was isolated with the High plasmid purification kit of Roche (REF: 11754785001). The centromere specific repetitive sequences CentBr1 and CentBr2 originate from *B. rapa* and were previously shown to paint the centromere regions of *B. oleracea* L. var. *botrytis* (Lim et al. 2005, 2007; Xiong and Pires 2011). These centromere-specific repetitive sequences were PCR amplified using the following primer sets:

CentBr1
forward primer: 5' -GAATAGCACAGCTTCATCGTCGTTCC-3'
reverse primer: 5' -CTGGGAAACTGTAATCACCTGATCTGAAA-3'

CentBr2
forward primer: 5' -GGGAATATGACACCTTCTTTGTCATTCT-3'
reverse primer: 5' -CAGGAAAACTGGGATCACCTGATTTAAAT-3'

Slide preparation

Slides were prepared as described in Chapter 2.

Fluorescence *in situ* hybridization

Probe DNA of the three repetitive sequences was either labeled directly with Diethylaminocoumarin-5-dUTP ('DEAC') (Perkin Elmer Life Sciences, Boston, MA, REF: NEL-455001EA) or indirect using the Dig-Nick-translation for FITC detection (Roche Applied Science, REF: 11745824910) or Biotin-Nick-translation for Cy5 detection (Roche Applied Science, REF: 11745816910) as descripted previously (Kato et al. 2004). The FISH protocol that we followed is essentially that of Chapter 2.

Immunodetection

We performed immunofluorescence microscopy with primary antibodies for detecting the mismatch repair protein MLH1, a protein marking class I crossover sites in spread pollen mother cells at meiosis. The MLH1 polyclonal antibody of *Arabidopsis thaliana* was obtained from Dr Liudmila Chelysheva (INRA, Versailles, France). Air-dried high quality acetic acid spread pollen mother cell slides were prepared as described in chapter 2. We heated 10 mM Na-citrate buffer (pH 6.0) in a microwave until boiling, submerged the slides in the boiling solution and cooked this in the microwave at 450W for 45s. The slides were then transferred to PBS-T buffer (1×Phosphate buffered saline, 0.1% v/v Triton, pH 7.4) and incubated for 5 minutes, followed by incubation of the slides with the MLH1 polyclonal antibody in PBS-T-BSA (1×PBS, 1%w/v BSA, 0.1% v/v Triton) in a dilution of 1: 200 at 4 ˚C overnight or over the weekend in a humid chamber. After incubation slides were washed in PBS-T for 3×15 mins. The secondary antibody Fluorescein (FITC)-conjugated AffiniPure F (ab')2 Fragment Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) was diluted in PBS-T-BSA (1:100) and used on the slides for 1 hour at 37 ˚C. Slides were then washed for 3×10 minutes in PBS-T, and then air-dried. Finally the slides were counterstained in 12 µL DAPI in Vectashield (50 µL/mL), and covered with a 24×50-glass cover slide. Microscopy and image processing was as described previously.

Statistical analysis

We selected mostly diakinesis / metaphase I cells and quantified the number of rod and ring bivalents, univalent pairs, chiasmata and chromosomes involved in interconnections. Basic statistical analyses (mean, standard deviation, ANOVA, Fisher's protected least significant difference test, etc.) were done using GenStat 64-bit Release 16.2, VSN International Ltd (http://www.vsni.co.uk/software/genstat).

Results

Description of the meiotic aberrations in the three lines

The basis of our analysis is the comparison of morphological and quantitative meiotic traits of three cauliflower lines that differ in rates of aberrant offspring. The so-called good line produces less than 5% aberrant plants in the offspring; the moderate one between 5 and 10% and the bad line has more than 10% offspring plants with aberrant phenotypes. For each of the three lines more than 100 DAPI stained pollen mother cells were observed. We focused mostly on cells at diakinesis (more than 50 cells per line were observed), and we also observed cells in pachytene, metaphase I, anaphase I and II, and tetrad cells.

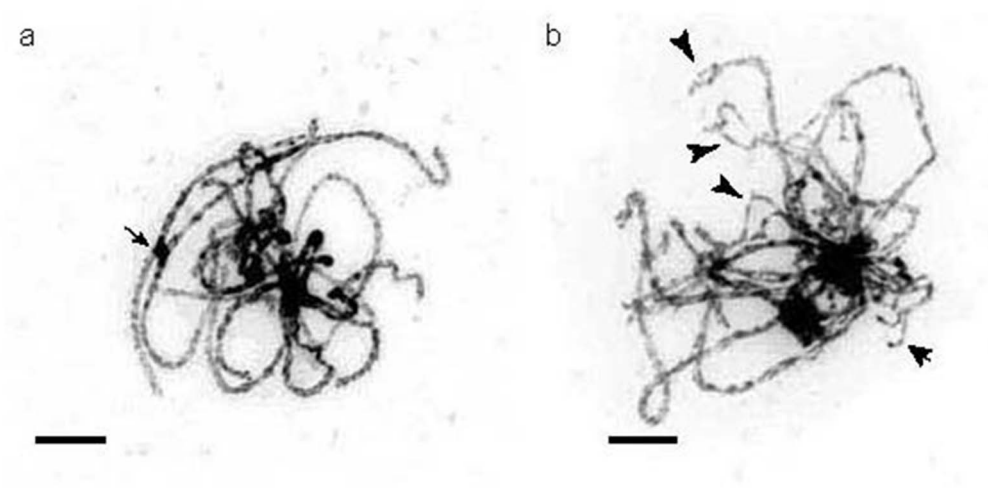

Figure 1. Photomicrographs of spread pollen mother cells of *B. oleracea* L. var. *botrytis* (bad line). The cells were stained with DAPI and the images inverted. Bars equal 10 µm. (a) Cell complement at late pachytene stage, in which homologous chromosomes are almost perfectly paired. The cells show a typical *Brassica* pachytene morphology: pericentromere regions are highly condensed large regions, which tend to cluster in a so-called synizetic knot. Some of the centromere or pericentromeres are connected (arrow) (de Jong, J. H. and P. Stam , 1985). The gene rich euchromatin has a large number of chromomeres (b) This cell complement is at late pachytene/ early diplotene, where NOR and other chromosome regions start to disjoin (arrow heads).

A conspicuous feature of pachytene chromosomes is the clustering of pericentromere heterochromatin, called synizetic knot. This is a general feature seen in many plants (Moens 1964) but is more pronounced in some *Brassica* species (Figure 1). The synizetic knot is thought to be a consequence of fixation in ethanol-acetic acid (Armstrong et al. 2001). The appearance of a synizetic knot made pachytene analysis a lot more difficult in *Brassica* species, however, besides the synizetic knot region, we observed no disturbance of homologous chromosome pairing at pachytene stages, and pairing was always complete (Figure 1a,b), except for a few small stretches. Such regions were considered as being at late pachytene, when some parts of the chromosomes start to detach (Figure 1b). We did observe some connections between some non-homologous centromeres or pericentromeres in a few cells in all three lines (Figure 1a), which resembles the previously described non-homologous association in meiotic prophase I of *Beta vulgaris* (de Jong and Stam, 1985).

At diakinesis, chromosomes are highly condensed and chiasma positions clearly distinguishable. We observed no, one or, sometimes, two chiasmata per chromosome arm (Figure

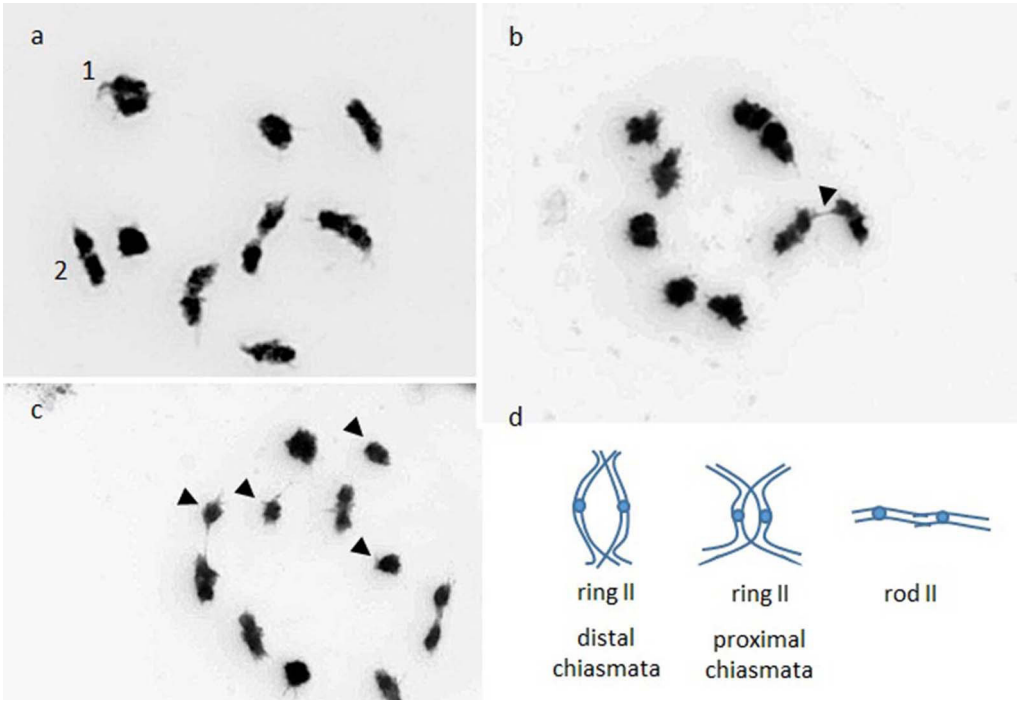

Figure 2. DAPI stained pollen mother cells of *B. oleracea* L. var. *botrytis* at diakinesis. (a) good line (b) good line (c) moderate line. (a) A ring-bivalent is formed when the two homologous chromosomes have at least one chiasma at the end of both chromosome ends (1. in the figure). In a rod-bivalent: chiasmata are confined to one of the chromosome arm ends (2. in the figure), (b) Example of a cell with a interconnection between two bivalents, (c) Univalent pairs: homologous chromosomes are not connected a one or more chiasmata, and hence remain separated as a univalent pair. (d) Schematic drawings of different bivalent types: ring bivalents have two chiasmata; the chiasmata may locate distally or proximally. A rod bivalent has one chiasma on one chromosome arm.

2d). Without a chiasma, the pair is a univalent pair and homologues are not attached. With one exchange between homologues, the bivalent attains the shape of a cross or that of a rod, depending on the position of the chiasma. With two chiasmata, a bivalent forms different shapes depending on proximal (close to centromere) or distal (close to the chromosome end) crossover positions. In most cells we observed ring and rod bivalents and univalent pairs, and very incidentally, putative trivalents and quadrivalents (Figures 2b, 2c, and Figure 3). Since the appearance of trivalents and quadrivalents is extremely rare, and these were found only after the statistics had been done, these cells are not included in our statistical analyses.

Most chromosomes pairs show at least one chiasma at diakinesis/metaphase I, but occasional univalent pairs occur in all lines and result from partial desynapsis. In the good line, univalents are rare and 10.5% cells contain univalent. Univalents are more frequent in the moderate and bad lines (39% and 42.3% respectively). In the moderate and bad lines we observed very incidentally (frying pan) putative trivalents and adjacent quadrivalents (Figure 3). We also observed interconnections between the homologous and non-homologous

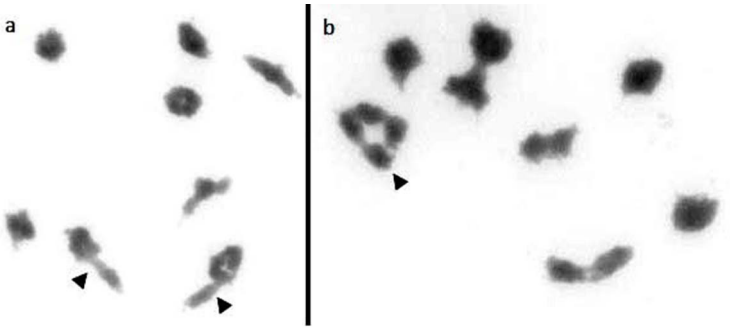

Figure 3. DAPI stained pollen mother cells of *B. oleracea* L. var. *botrytis* at diakinesis showing exceptional configurations (moderate line), (a) two sets of three chromosomes are identified that are associated through chiasmata forming a frying pan trivalent (b) Four chromosomes connected by chiasmata forming a quadrivalent (arrow). In the second set of 4 chromosomes it is not clear from the image if all chromosomes are connected by chiasmata.

chromosomes in all lines. In some cells of the moderate and bad line, nearly all bivalent pairs were connected with each other.

At metaphase I one expects the alignment of nine bivalents with their chiasmata at the equatorial plate and centromeres facing the poles. However, several aberrations were observed, including the rare multivalents as mentioned above (Figure 4a). At anaphase I, the half bivalents segregate normally, whereas the univalents segregate randomly resulting in unbalanced chromosome numbers at the poles. Moreover, in the moderate and bad lines, we observed anaphase bridges and multiple lagging chromosomes (Figures 4b and 4c). Also at anaphase II, we observed anaphase bridges and chromosome stickiness (bivalent interconnections) (Figures 4c and 4d). In tetrad stage cells, we found regular tetrads (9+9+9+9)

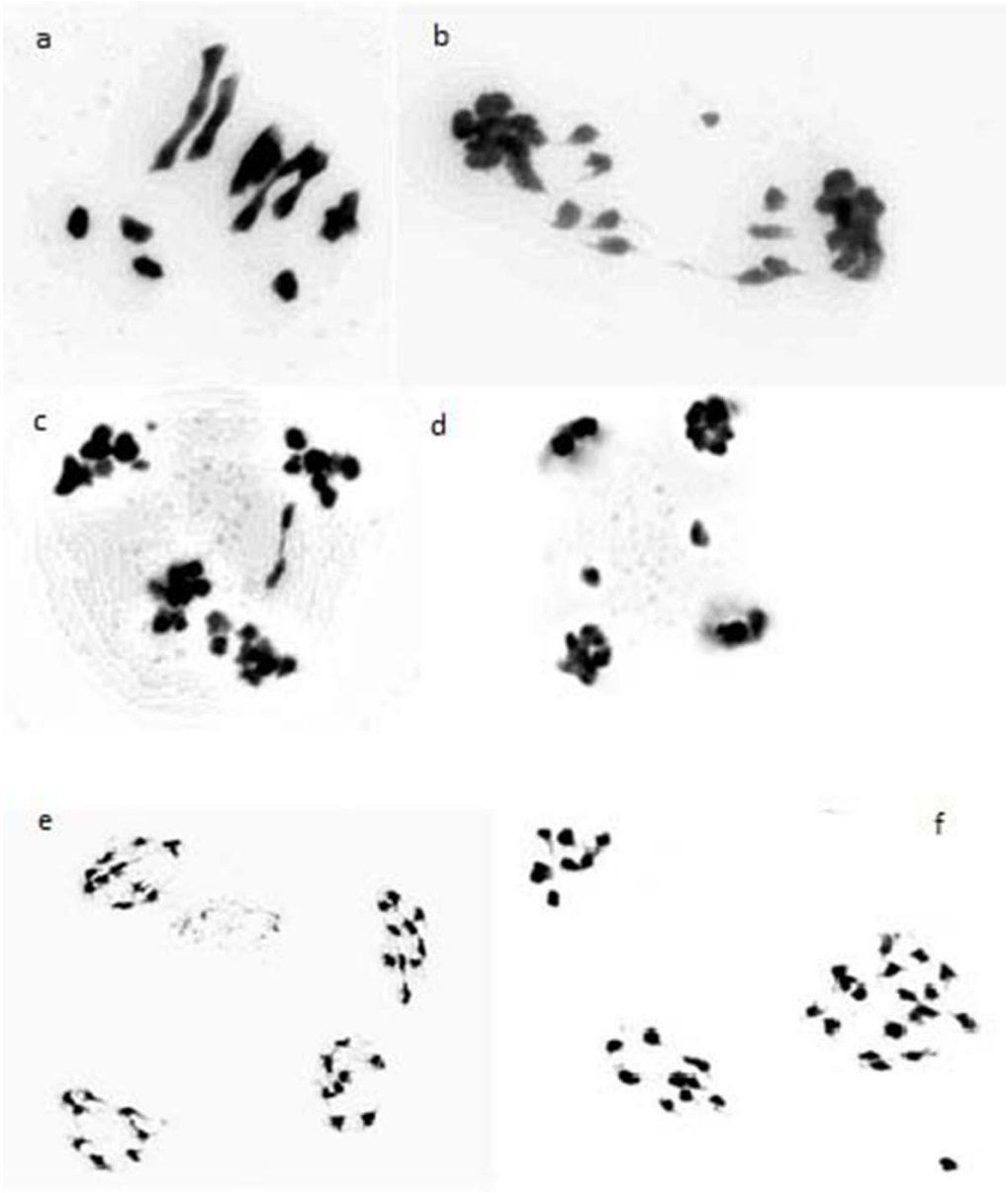

Figure 4. DAPI stained pollen mother cells of *B. oleracea* L. var. *botrytis* at later meiotic stages (a) (b) (e) bad line, (c) (d)(f) moderate line, (a) Metaphase I/Anaphase I: univalents have not congressed to the equatorial plane, and their segregation to the poles with lead to unbalanced gametes, (b) Anaphase I with laggards and fragments. (c) Anaphase II: bridges were observed. (d) Anaphase II: ana-phase II with laggards. (e)+(f) Unbalanced tetrad, unbalanced gametes may form from these tetrad (e) 10+10+8+8, (f) 9+9+9+8+1

Table 1. Numbers of rod-, ring- and univalents per cell, including chiasma estimates and bivalent connections among three lines.

	good line	moderate line	bad line
number of cells	57	59	52
ring bivalents	303	295	288
rod bivalents	201	203	152
univalent pairs	8	32	28
number of chiasmata	807	691	728
bivalent connection (stickiness)	53	115	109
ring bivalents per cell	5.3	5	5.5
rod bivalents per cell	3.5	3.4	2.8
univalent pairs per cell	0.14	0.54	0.54
number of chiasmata per cell	14.10	13.46	13.80
bivalent connections per cell	1.10	1.95	2.09

in the good line, but in the moderate and bad lines we also found other chromosome distributions such as (9+9, 9+8+1) and (10+8, 10+8) (Figures 4e and 4f).

Univalents occur in higher rates in the moderate and bad line

The occurrence of univalents, rod bivalents and ring bivalents at diakinesis cells are summarized in table 1 for all three lines (cf. Figure 2). The percentages of cells showing an expected number of nine bivalents are 89%, 61% and 57% for the good, moderate and bad line respectively. In most of the cells which contained univalent pairs only one univalent pair was present, while in the moderate and bad lines, two or more pairs of univalents were regularly observed in one cell. In the good line, 7% of the cells have one pair of univalents and 4% of cells have two pairs of univalent. In the moderate line, 27% of the cells have one pair of univalents and 12% of cells have two pairs of univalents. In the bad line, 31% of the cells have one pair of univalents and 12% of cells have two pairs of univalents. To determine whether the three lines differ significantly in univalent incidence, we applied a one way ANOVA test on univalent counts. The results suggests that the number of univalent pairs differs significantly between the three lines (Table 2, figure 6c, figure 8). Using Fisher's protected least significant difference test, we found that the good line differs significantly from both the moderate and bad line, while the moderate and bad lines do not differ from one another (Table 3, figure 6c, figure 8).

Crossover estimates in the three lines

Because the moderate and bad lines show higher numbers of nonrecombining chromosomes, the naïve assumption would be that these lines show lower total levels of crossover recombination. We therefore estimated the total number of crossovers based on the numbers of counted rod and ring bivalents (accounting for 1 and 2 crossovers respectively, Table

Table 2. ANOVA analysis of univalent pairs in the three lines.

Source of variation (univalent)	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	2	6.0319	3.0159	6.68	0.002
Residual	165	74.4443	0.4512		
Total	167	80.4762			

P-value is 0.002<0.05; it indicates a significant difference in number of univalent pairs between the lines. (d.f.: degrees of freedom, s.s.: sum of squares, m.s.: mean square, v.r.: variance ratio, F pr.: p-value).

Table 3. Fisher's protected least significant difference test on univalent prevalence among all cells observed in three lines.

Comparison (univalent)	Difference	Lower 95%	Upper 95%	t	Probability	Significant
good line vs moderate line	-0.3981	-0.7028	-0.0935	-3.091	0.0023	Yes
good line vs bad lline	-0.402	-0.6971	-0.107	-3.223	0.0015	Yes
moderate line vs bad line	-0.0039	-0.3061	0.2983	-0.031	0.9756	No

The good line is significantly different from the moderate and bad lines: probability: 0.0023 < 0.05 and probability: 0.0015 < 0.05, while the moderate and bad lines do not differ significantly in univalent frequency (probability: 0.9756 > 0.05).

Table 4. ANOVA analysis of crossover number among all cells observed in three lines.

Source of variation (crossovers)	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	2	15.898	7.949	1.91	0.152
Residual	165	688.477	4.173		
Total	167	704.375			

P-value 0.152 > 0.05; it indicates no significant differences in crossover number between the lines.

1, Figure 1a and Figure 6a). The average crossover numbers in the good, moderate and bad line were 14, 13 and 14 per cell. Perhaps superfluous, an ANOVA analysis confirms this is not statistically different (Table 4). We therefore conclude that desynapsis (univalent formation) does not decrease the total numbers of crossovers.

The mechanism leading to desynapsis is unknown. If desynapsis is a late event (*i.e.*, failure to stabilize a chiasma during pachytene/diakinesis), one would, as mentioned above, assume that crossover numbers decrease. But we found that this is not the case. One could coin an alternative hypothesis, in which desynapsis results from an earlier event, where the failure to establish a crossover on a specific chromosome pair, leads to higher crossover numbers on the remaining chromosome pairs. This would lead to the prediction that those cells with univalent pairs have higher crossover numbers (i.e. then this would be a cellspecific effect). We therefore compared the average crossover number in cells with univalent

pairs with the average crossover number in cells that did not display univalents. Our data show that the presence of univalent pairs indeed leads to an increase of recombination on other chromosome pairs within the cell complement (Tables 5 and 6). While the total num-

Table 5. Average number of ring and rod bivalents and chiasmata in cells without univalents.

per cell (normal cell)	good line (n=51)	moderate line (n=36)	bad line (n=30)
ring bivalents	5.5	5.4	5.8
rod bivalents	3.6	3.6	3.2
total number of chiasmata	14.6	14.4	14.8

Table 6. Average number of ring and rod bivalents in cells with univalents.

per cell (cells contained univalent)	good line (n=6)	moderate line (n=23)	bad line (n=22)
ring bivalents	3.2	4.3	5.2
rod bivalents	4.5	3.2	2.5
total number of chiasmata	10.9	11.8	12.9

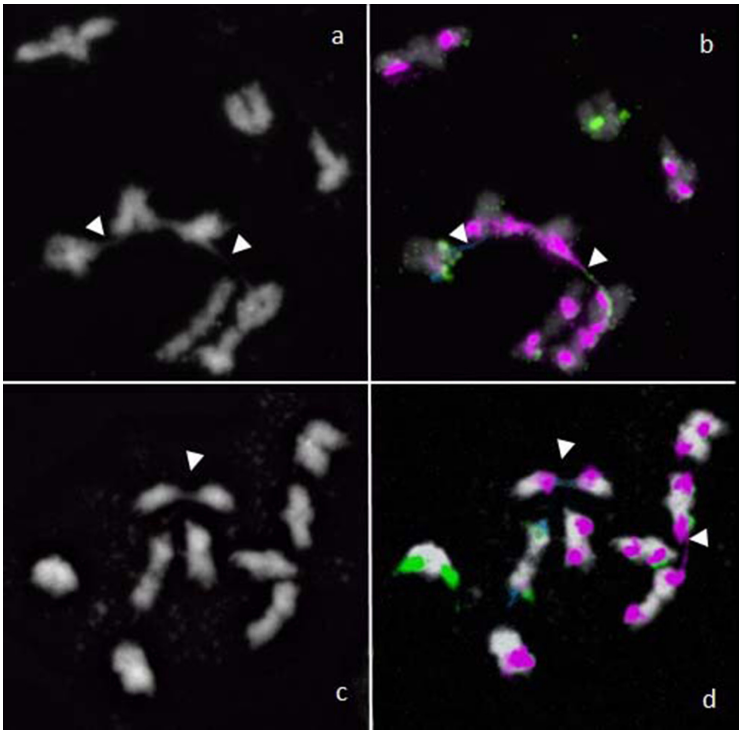

Figure 5. FISH with labeled repetitive sequence FISH on spread pollen mother cells of *B. oleracea* L. var. *botrytis* at diakinesis. Purple fluorescence: Centbr1, green fluorescence; Centbr2, blue fluorescence; 45S rDNA. (a) + (c) DAPI staining clearly showing the interconnections; (b) + (d) FISH detection with two centromere repeat and 45s rDNA, the connections were painted by repetitive sequences.

Table 7. ANOVA analysis of bivalent interconnections in the three lines.

Source of variation (bivalent interconnection)	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	2	45.194	22.597	15.09	<.001
Residual	165	247.086	1.497		
Total	167	292.28			

Table 8. Fisher's protected least significant difference test on bivalent interconnections among three lines

Comparison (interconnections)	Difference	Lower 95%	Upper 95%	t	Probability	Significant
good line vs. moderate line	-1.0193	-1.557	-0.4818	-4.485	<.001	yes
good line vs. bad lline	-1.1663	-1.721	-0.6113	-4.97	<.001	yes
moderate line vs. bad line	-0.147	-0.698	0.4035	-0.632	0.5286	no

The good line is significantly different from the moderate and bad lines: probabilities are less than 0.05, while the moderate and bad lines do not differ significantly in bivalent interconnections (probability: 0.5286>0.05).

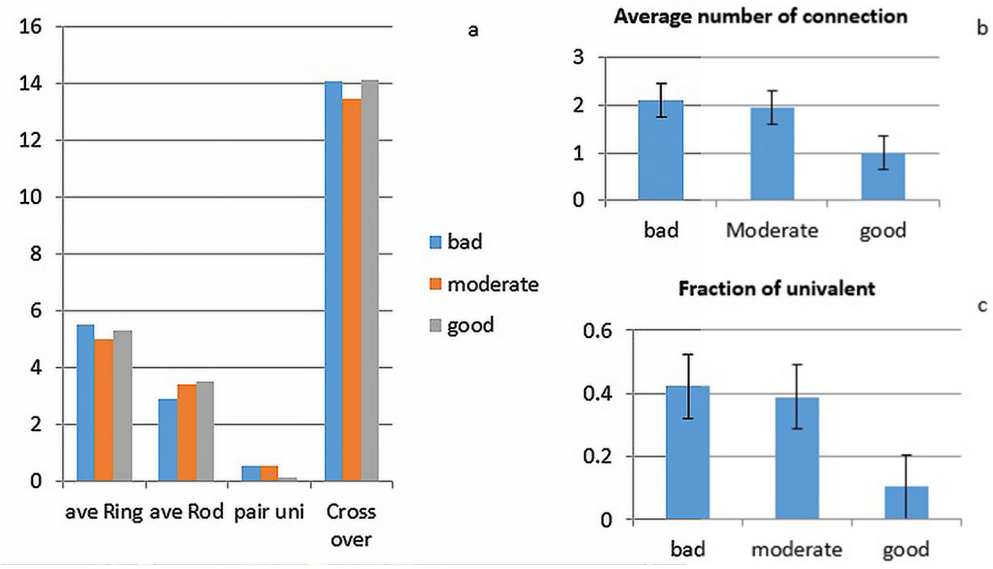

Figure 6. (a) Number of ring and rod bivalents, univalent pairs and crossovers for each cell in the three cauliflower lines. The bad and moderate lines have more univalent than the good line. (b) Average number of interconnections per cell in three cauliflower lines: bad and moderate lines have more interconnection than the good line. (errrorbars: standard error) (c) Percentage of univalents in three lines: the bad and moderate lines have around 40 % cells containing univalents; while the good line has around 10% cells that contain univalents. (Errrorbars: standard error).

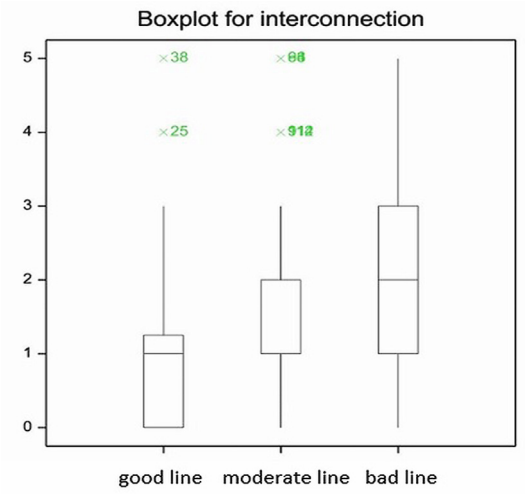

Figure 7. Boxplot for interconnection per cell between the three lines. The vertical line for each boxplot represent maximal and minimal numbers of interconnections for each cell with exceptional outliers indicated by green crosses (with whiskers with maximum 1.5 IQR). Good line: most cells contain 0-3 interconnections while one cell contains 4 interconnections and one cell contains 5 interconnections (green crosses), the median number of interconnections is 1 per cell. Moderate line: cells contain 0-3 interconnections; and three cells contain 4 interconnections and four cells contain 5 interconnections (green crosses) (indicated by green crosses), the median interconnection is 2 per cell. Bad line: cells contain 0-5 interconnections; the median number of interconnections is 2 per cell.

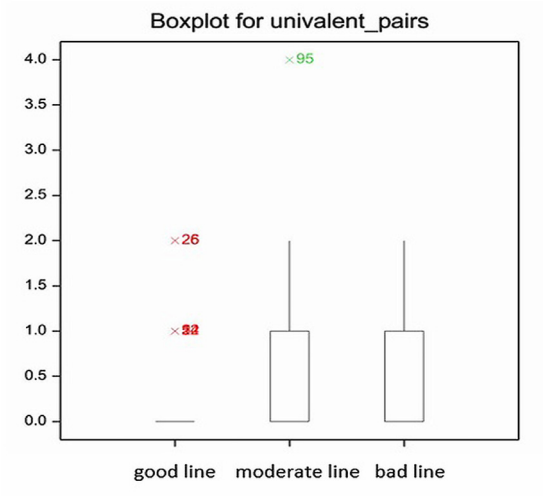

Figure 8. Boxplot for univalent numbers per cell between the three lines. The vertical line for each boxplot represent maximal and minimal numbers of univalents for each cell, red or green crosses indicate outliers (with whiskers with maximum 1.5 IQR). Good line: most cells contain no univalents while four cells contain 1 univalent and two cells contain 2 univalents (indicated by a red cross). Moderate line: cells contain 0-2 univalents; one cell has four univalents (indicated by green cross), the median number of univalents per cell is 1. Bad line: cells contain 0-2 univalents; the median univalent number is 1 per cell.

Table 9. Number of MLH1 foci per cell among three lines.

	good line (n=15)	moderate line (n=53)	bad line (n=35)
MLH1 foci (per cell)	10.93	10.43	9.52

ber of observations is not that high, our data are highly suggestive of such a mechanism. These observations suggest that desynapsis in *Brassica* finds its origin in early prophase.

Bivalent connections (stickiness)

Besides univalent formation, the presence of bivalent connections is another remarkable feature of *Brassica* meiosis. We counted numbers of connections between bivalents (Figure

5a, 5c; Table 1). The average numbers of connections were 1.1, 2.0 and 2.1 in the good, moderate and bad line respectively.

A one-way ANOVA suggests these differences are significant (Table 7, figure 6a), and a subsequent Fisher's protected least significant difference test indicates the good line differs significantly from both the moderate and bad line. There is no significant difference between the moderate and bad line (Table 8; Figure 7).

Immunodetection of chiasmata

There are two known molecular pathways leading to crossover formation in plants, known as the class I and class II crossover pathways. We wondered whether univalent formation resulted from a dysfunction in one of the pathways. We therefore did an immunofluorescent detection of MLH1 in diakinesis cells, which stains class I crossover sites and typically comprise an estimate 85% of all crossovers in plants. MLH1 foci clearly corresponded to the sites of expected crossovers (Figure 9), but were lacking at other places where chiasmata were apparent. These are presumed sites of class II crossovers. We observed at least 15 cells for each line, and the results indicated the number of class I crossover in the good, moderate and bad line were 10.93, 10.43 and 9.52 respectively (Table 9). Intriguingly, we observed MLH1 foci at some bivalent connections (Figure 9c).

Repetitive DNA FISH analysis of diakinesis / metaphase I

We suspected there might be repetitive sequences involved in bivalent connections, as was previously suggested by Pedrosa et al. (2001) based on observations in *Ornithogalum longibracteatum* (*Hyacinthaceae*). To test this, we performed a FISH experiment with repetitive DNA. 45s rDNA and two *Brassica* specific centromere repeats were used as probes. We found that 45s rDNA as well as the two *Brassica* centromere repeats painted bivalent connections. Around 70% of bivalent connections involved centromere and 45S rDNA specific sequences, while less than 30% of the connections comprised unstained chromatin. When 45s rDNA connections were observed, one or both of the chromosomes involved carry a 45s rDNA locus, whereas when centromere repeats are involved, these connect the centromere regions of two homologues (Figures 5b and 5d).

To evaluate whether chromosomal interconnections are random, or whether specific chromosomes are more often involved in interconnections, we used a subset of 19 painted interconnections that comprised 45s rDNA. Cauliflower has 9 chromosome pairs, two of which have 45s rDNA loci. Connections between chromosomes with 45s rDNA (45S-45S), between a chromosome with and without a 45s rDNA signal (45S-non) and between chromosomes without 45s rDNA signals (non-non) were quantified (see Table 10). The theoretical chances of connections between chromosomes (while assuming no preferential

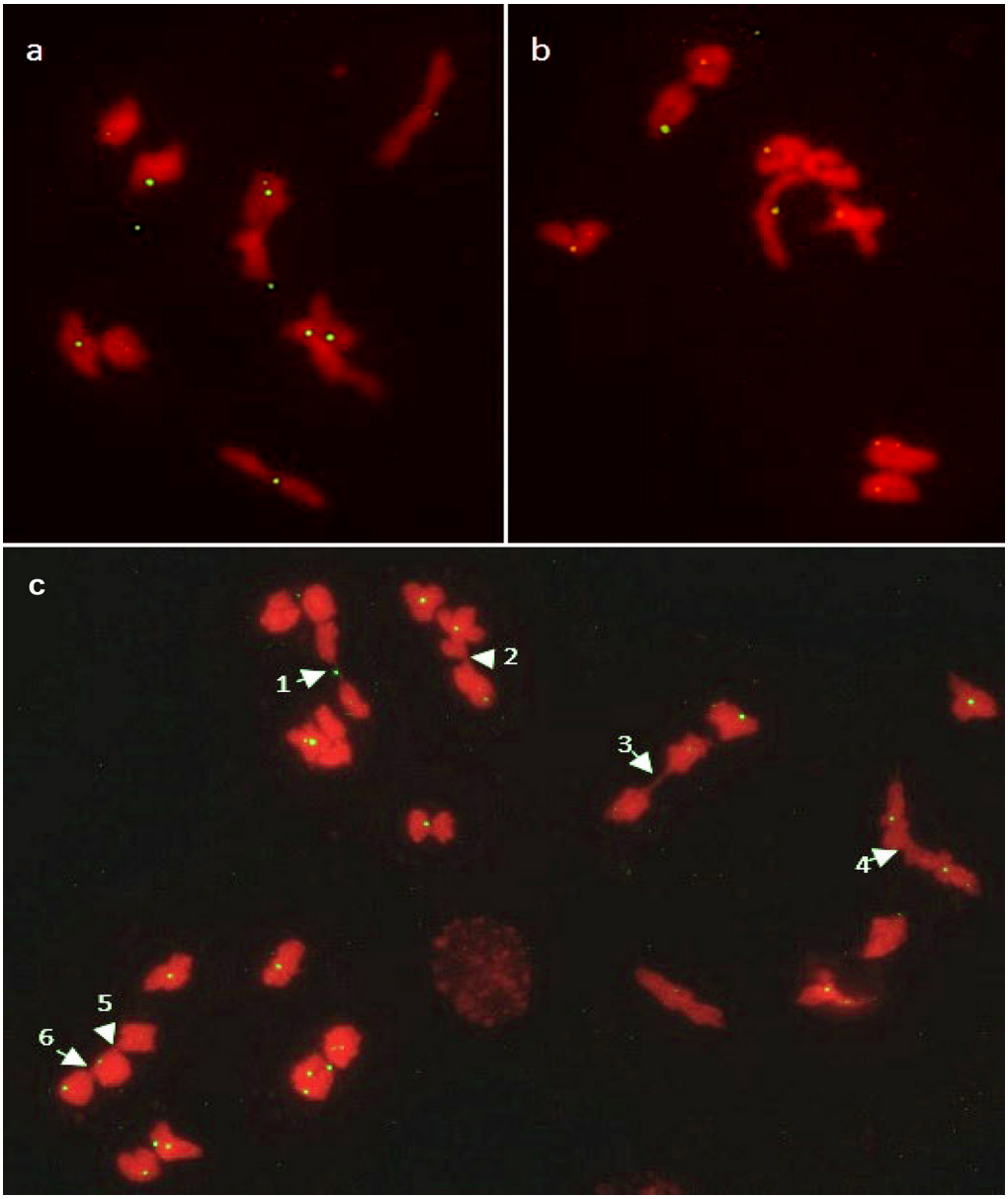

Figure 9. MLH1 immunofluorescence (a) bad line, (b) (c) moderate line. MLH1 detected crossover foci at diakinesis. Since MLH1 does not stain all crossovers, some crossover sites have no signals. (c) arrows indicate bivalent connections at diakinesis. Most of these do not contained MLH1 foci, except arrow 1 which indicates a MLH1 focus at a bivalent connection.

Table 10. The localization of 19 bivalent interconnections that comprised 45s rDNA. See text for explanation.

Connection	observed	Expected
45S-45S	4	0.513
45S-non	10	7.41
Non-non	5	11.02

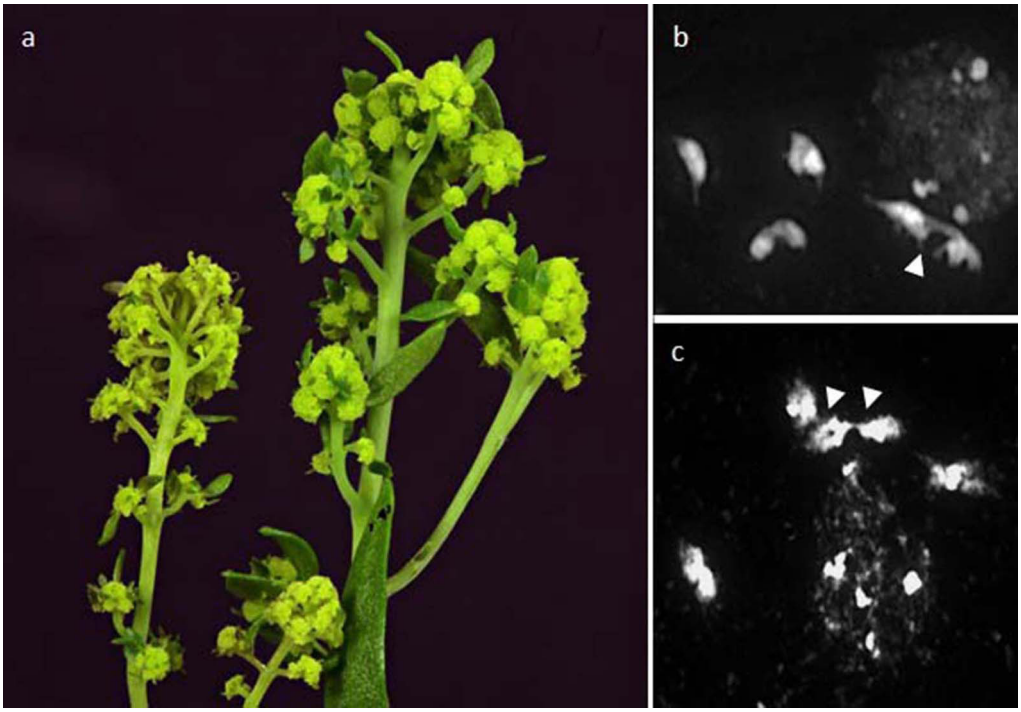

Figure 10. (a) The AP1/Cau double mutant of Arabidopsis forms a typical curd phenotype resembling that of cauliflower. (b) + (c) microscopic study with meiotic pollen mother cell of *AP1*/*Cau* mutant: Pollen mother cells at diakinesis. Bivalent interconnections between bivalents are clearly observed whereas univalent pairs are completely lacking.

connections being formed) are given by the following chances: P(45s-45s)= 2/9*1/8 =1/36 , P(45S-non) = 2/9*7/8+7/9*2/8 = 14/36 and P(non-non) = 7/9*6/8 =21/36. We used a χ^2-test to compare observed and expected data. With a χ 2 value of 27.8, and pdf=2< 0.001, a significant over representation of 45S rDNA carrying chromosomes in bivalent connections is clear (Table 10). At least for the 45s rDNA, the interconnections are not random, but preferentially occur between chromosomes that have functionally similar regions.

Meiotic analysis result of an *APETALA1/ CAULIFLOWER* mutant in *Arabidopsis*

Since cauliflower is of all Brassica crops most plagued by aneuploidy in its offspring, we wondered whether the genes responsible for curd formation may indirectly cause aneuploidy among offspring. We therefore studied the *APETALA1/ CAULIFLOWER* double mutant of *Arabidopsis* that, like cauliflower, displays the typical curds (Figure 10a). Interestingly, pollen mother cells at diakinesis in the *Arabidopsis* double mutant showed bivalent interconnections (Figure 10b), albeit in far lower numbers than *Brassica*: in less than 10% of all cells. Univalents were not observed nor did we find evidence for aneuploid offspring.

Discussion

In this study we set out to explore whether the occurrence of aneuploid offspring among cauliflower progeny might be attributed to the occurrence of irregularities in meiosis. The underlying assumption would be that aneuploid gametes are the most likely source of aneuploids in the offspring. We therefore analyzed meiosis in three cauliflower lines that differ in the percentages of aneuploid offspring they produce. We hereafter will firstly discuss meiotic progression in brassica, with a discussion of the main anomalies of *Brassica* meiosis after which we address the effects this might have on breeding in Cauliflower.

Aberrations in cauliflower meiosis: crossover formation

Our observations of Cauliflower meiosis show that the chromosomes at pachytene pair regularly. However, at diakinesis, univalents become visible and bivalent connections were found to be present in all three lines. Univalents were found to occur in all lines, but have a much higher incidence in the moderate and bad lines (as these were seen in about 40% of meiotic cells). Most of the cells show one univalent pair, but in the moderate and bad lines, two or even four pairs of univalents were observed. The combination of pachytene pairing with the emergence of univalent pairs at the end of meiotic prophase is known as a desynaptic phenotype.

The presence of univalents leads to unbalanced chromosome segregation. In tetrad stage cells we found different tetrad combinations such as (9+9, 9+8+1), and (10+8, 10+8) in the moderate and bad lines, rather than the regular, expected numbers (9+9+9+9) (Figure 4e, 4f). The gametes with chromosome numbers less than nine will not be fertile, while those with chromosome numbers higher than nine, may cause trisomies (aneuploidy).

The desynaptic phenotype of meiosis in cauliflower lead us to assume that the total amount of meiotic recombination would go down. But counts of crossover numbers in cells showing pairs of univalents showed that the number of crossovers in such cells is similar as in cells in which all chromosomes are joined by chiasmata. In other words, the loss of cross-

overs by univalent pairs is apparently compensated by higher numbers of crossovers on the bivalent pairs in the same cell complement (tables 5 and 6).

Aberrations in Cauliflower meiosis: bivalent connections

Apart from aberrations in crossover formation, cauliflower shows the presence of bivalent connections (chromosome stickiness) in all lines. At least one bivalent connection was observed in each of cells in cauliflower meiosis, while in the moderate and bad lines an average of two bivalent connections was observed. From our BAC FISH results, the centromere and 45s rDNA repetitive sequences seem to be regularly involved in bivalent connections, where we showed that 45S rDNA carrying chromosomes are more regularly interconnected than expected by chance. Furthermore, when centromere sequences were involved, these always connected the centromere regions of different homologue pairs.

Although the cause for these interconnections is unknown, we found that functionally similar regions tend to interconnect between homologues. We assume that the bivalent connections are caused by erroneous DNA repair as a from meiotic double strand breaks. Or observation of MLH1 foci at the sites of some bivalent interconnections is highly indicative of the meiotic recombination machinery being involved in these interconnections (figure 9c). MLH1 foci result from double strand break repair in the class I crossover pathway, which result from homologous recombination (Dion et al., 2007). Together with the observations that many bivalent interconnections involve repetitive sequences, our dataset is highly suggestive that DSB repair in *Brassica* results from ectopic homologous recombination, likely involving repetitive sequences.

Chromosome stickiness has been described to occur in interspecific hybrids as well as in inbred lines and may be caused genetic mutation or environmental factors, which later leads to unequal distribution of genetic material to the daughter cells (Rayburn and Wetzel, 2002). It may give rise to a higher frequency of non-disjunction, unbalanced segregation at anaphase, increase the frequency of translocations and the rate of gene mutation (Beadle, 1933) and abnormal chiasma formation (Higgins et al. 2005). Furthermore, chromosome stickiness has been reported to lead to dicentric anaphase bridges (Basi et al. 2006). Our observations of anaphase bridges in meiosis I and II in cauliflower meiosis thus concur well with previous observations (figure 4b and 4c).

What causes meiotic aberrations in Cauliflower?

Among all B. oleracea varieties, cauliflower (B. oleracea L. var. botrytis) is most susceptible to aneuploid formation. Since cauliflower has a huge curd as a unique phenotype in B. oleracea varieties, we hypothesized that domestication of cauliflower curd may have introduced the desynaptic phenotype. To test our assumption, we chose the *APETALE*1 / *CAULIFLOWER* double mutant of *Arabidopsis thaliana* as a model, since this double mutant

forms a curd phenotype like cauliflower. We found bivalent interconnections in the double mutant, but the observation of bivalent interconnections in the double mutant is less than 10%, and no observation of univalent pairs, or aneuploid progeny. The apetala1 / cauliflower double mutant shows a curd phenotype when in homozygous recessive form (Bowman et al. 1993). Chromosome stickiness has not been described for this mutant before, and no interactions with the DNA repair machinery are known.

Desynaptic mutants have been described for a number of species (Cai and Makaroff 2001, Jauhar and Singh 1969, Rao 1975). An interesting paper was written by Higgins et al. (2005) who described the phenotype of zip1 mutants in *Arabidopsis*, which shows a number of phenotypes that are concurrent with what is seen in cauliflower: the formation of univalents, chromosome stickiness and crossovers between non-homologous chromosomes. When the expression of ZIP1 is impaired, no chromosome pairing is observed in these cells, and if meiosis in Cauliflower would be caused by problems with ZIP1, we would have to assume a hypomorphic phenotype. Whether zip1-like mutations are involved in the cauliflower mutant phenotype cannot be confirmed.

Although we noted the occurrence of trivalents and quadrivalents, we did observe a few. Trivalents and quadrivalents were observed in the moderate and bad lines. Trivalents were found in many species as potato (Singh et al. 1988, Wagenvoort 1995), maize (Maguire 1970) and rye (Díez et al. 2001). Quadrivalents are mostly observed in hybrids or allopolyploids, as Avena (Ellison 1938), or may result from translocation heterozygosity (Loidl 1995). As our cauliflower material is diploid and not hybrid, quadrivalent formation in Brassica is the presumed result of non-homologous pairing and recombination since there are only two homologues present for each chromosome.

Does meiosis cause aneuploidy?

The three lines we studied were known to differ in the amount of aneuploids found in their offspring. Our good line, which produces the smallest percentage of aberrant offspring, also shows the lowest amount of univalents and stickiness, has as such a more regular meiosis and produces the highest number of balanced pollen. The moderate line, although it produces less aberrant progeny than the bad line, shows a much more disturbed meiosis than the good line. It has a higher incidence of univalent formation and shows more bivalent connections. The bad line is equally bad as the moderate line in terms of meiotic aberrations. An increase in meiotic aberrations in the parental lines thus correlates quite well with the occurrence of aneuploid offspring. Such would also be expected, as the more chromosome segregation is disturbed, the more unbalances gametes are formed.

Chable et al. (2009) suggested that aneuploidy cannot be related to aberrant phenotypes in cauliflower parents, but our results suggest, at least in part, that this is not the case. However, we cannot explain the higher numbers of aneuploidy offspring in the bad line. A reason for this might be that we observed male meiosis, while female meiosis that is much more

difficult to study, will likely contribute to the observed generation of aneuploidy offspring. Koornneef and van der Veen (1983) studied the transmission of trisomies between female and male meiosis in *Arabidopsis*, and showed that trisomies are more likely to be transmitted through female meiosis. It might be that the bad line is more strongly affected in female meiosis than the moderate line.

According to Klášterská (1975, 1976), chromosome stickiness can vary very much, such that in the same plant degenerating and normal cells may both appear. If environmental factors would influence stickiness, it could well affect the balanced segregation of chromosomes due to environmental factors. Is should be noted though, that we cannot ascertain that chromosome stickiness leads to aneuploidy in cauliflower. Aneuploidy would equally well be explained by the occurrence of univalents only. As the *apetala*1 / *cauliflower* double mutant shows (albeit low) numbers of bivalent interconnections, and no aneuploidy, it might be speculated that bivalent interconnections do not contribute to aneuploidy in curd-forming mutant plants.

Future perspective

In this research, we observed cauliflower male meiosis, and we conclude that aneuploidy formation in cauliflower is likely caused by univalent formation which becomes evident after the pachytene stage in meiosis. An interesting further research question is whether female meiosis is equally affected as male meiosis, or whether it would even explain further differences between the lines in terms of the observed numbers of aneuploids in their offspring. Furthermore, we would like to know if univalents and chromosomal connections also occur in cauliflower female meiosis like in *dmc*1 mutants of *Arabidopsis thaliana* or *ds*-1 mutant of potato (Couteau et al. 1999, Jongdijk et al. 1990), or whether there are other meiotic problems like non-disjunction, loss of sister-chromatid cohesion, stickiness, first and second division restitution (Jongedijk et al. 1991, Cai and Xu 2007).

For breeders, it is important to develop a workable strategy to measure pollen fertility as a measure of balanced gamete formation to select for lines that produce normal euploid pollen. The method that is mostly applied is pollen observation, in which pollen are stained with staining solutions like acetocarmine or lactophenol acid fuchsin. However, aneuploidy is difficult or impossible to detect using this method. Eskilsson (1963) suggested a staining method to estimate fertility of autopolyploid plants, the method uses with paraffin oil as an embedding medium after which pollen quality can be assessed. By applying these methods on *Trifolium pratense* pollen grains, it could be shown that diploid pollen grains from tetraploids showed more variations in size and shape with paraffin oil medium than with common staining methods. Measuring DNA content in pollen with flow cytometry may be another method for determining unbalanced gametes. Kron and Husband (2012) applied this method to estimated unreduced gametes, according their result flow cytometry dra-

matically improved the estimation of unreduced gametes, When optimized, such methods might be applied to cauliflower as well.

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Chapter 5

Cytogenetic characterization of a paracentric inversion on chromosome arm 4S of *Arabidopsis thaliana*

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Abstract

A heterochromatin knob of 1,170 kb on the short arm of chromosome 4 has been found in the Columbia accession of *Arabidopsis thaliana*, whereas it is absent in Landberg erecta. The knob was identified as a relocated pericentromere heterochromatin segment caused by an ancestral paracentric event. BAC-FISH painting and comparative genomics of the Col and Ler sequences in that region positioned the borders of this inversion at 1,612kb and 2,782kb from the NOR, respectively. Genetic analysis of an F1 hybrid formed between the Col and Ler accessions revealed complete absence of genetic recombination in the region heterozygous for the inversion. Subsequent Fluorescent in situ Hybridization (FISH) analysis of pooled BACs covering the proximal and distal parts of the inversion confirms that the corresponded chromosome segments have opposite orientation. We did not observe an inversion loop at pachytene and only rarely we detected a chromosome bridge at anaphase I. In addition, immunofluorescence with antibodies to the Asy1 and Zip1 synaptonemal proteins displayed a discontinuity in the inverted region of Zip1, which confirmed lack of synapsis and crossover formation in that region. We next included 30 other accessions of *Arabidopsis* for our study on the inversion. DAPI stained pachytene complements were analysed for the presence of the heterochromatic knob in chromosome arm 4S, whereas primers were designed around the proximal and distal inversion breakpoints for a PCR assay to validate the presence of the inversion. There was a perfect match between the occurrence of the knob and the PCR based proof for the inversion. We also compared SNPs in and around the inversion and observed that the knob carrying accessions have very few SNPs in the inversion compared to the Columbia reference and that accessions may differ in their borders of the SNP empty region suggesting that they arose from independent ancestral recombination events flanking the inversion breakpoints.

Introduction

Eukaryotic genomes can evolve through a combination of processes that are associated with clear changes in chromosome number, morphology and organization. Most dramatic events follow nuclear restitution or unequal chromosome segregation leading to loss or gain of complete genomes or chromosomes, whereas breakage and ligation of chromosomal segments can lead to micro-rearrangements or to gross karyotype and chromosome structure changes such as inversions, translocations, centric split and fusion, duplications and deletions. Numerous comparative cytogenetic studies on chromosomal rearrangements are known from the Drosophila literature (e.g., Hoffmann et al., 2004; Bhutkar et al., 2008; Kirkpatrick, 2010; Song et al., 2011), Anopheles spec. (Coluzzi et al., 2002; Sharakhov and Sharakhova, 2010; Xia et al., 2010) and other dipteran containing displaying highly detailed polytene banding patterns. In plants both cytogenetic studies and linkage analyses revealed large numbers of numerical aberrations and structural variants but only few show inver-

sions in great detail (Laufs et al., 1999; Szinay et al., 2012), but could not detect smaller changes in the chromosome structure down to the DNA level, neither could they establish the consequences for such events for meiotic recombination, introgressive hybridizations or fixation rates in natural populations.

The wealth of high-throughput genome technologies and dense SNP maps in various model species has provided recently a tremendous number of data on detecting structural rearrangements (Feuk et al., 2006; Faraut 2008). In addition, molecular cytogenetic tools including multicolour FISH (Fluorescent in situ Hybridization) with isolated chromosome specific DNA or BACs (Bacterial Artificial Chromosomes) or smaller vectors as probes on chromosomal targets (Wienberg and Stanyan 1997, Müller et al, 1999, Rocchi et al., 2012) or DNA fibres (e.g., Florijn et al. 1995) demonstrated structural chromosome variants that hitherto were not detected by any other method. Although initially restricted to comparative studies in human, primates and other mammal species the technologies have now also widely been applied to plant species as well. Lysak et al. (2001, 2003, 2006, 2010) used the BAC FISH painting to detect manifolds translocations in the Brassica family, whereas various labs described numerous inversions in crops and wild species of the Solanaceae family (Iovene et al. 2008; Tang, Szinay et al., 2008; Lou et al., 2010; Anderson et al., 2010; Szinay et al., 2010b; Wu and Tanksley, 2010). Where complete de novo assembly of related species was obtained multiple genome alignment technologies including MUMmer plot analyses revealed unprecedented comparative genomics revealing inversions, translocations and other structural variants (Kurtz et al., 2004; Pop et al., 2004; Ohtsubo et al, 2008; Cheung et al., 2009; Darling et al., 2010). Such studies also have the benefit of producing essential information about the molecular organization of the breakpoints involved in the rearrangements, and so can shed light on the origin of the rearrangements and the eventual involvement of transposons and other repeats, and the role of epigenetics in releasing such repeats if any.

Inversions in general have little or no consequences for regulation and expression of genes if their breakpoints do not alter gene function. However, heterozygosity for the inversion will prevent homologous regions to pair in that region and eventual formed crossovers in the inversion loop will lead to unviable recombinant chromosomes. As a consequence genes in the inverted region will not recombine and hence inherit as a single locus. In natural populations inversions seem the most prevalent large scale structural chromosome variant and are found in subspecies, accessions and related wild genotypes (Madan, 1995), but large-scale recordings of natural polymorphisms were – apart from Drosophila (Bhutkar et al., 2008), Anopheles (Lobo et al., 2010) and human populations (Bansal et al. 2007; Stefansson et al., 2005; Feuk et al., 2006) – only described in few species due to the lack of fast and efficient inversion detection methods.

Despite the many genetic and cytogenetic studies on inversions of related plant species very few of them report the occurrence of inversion polymorphism at the intraspecific level. Fransz et al. (1998) and Koornneef et al. (2003) described a small heterochromatic knob in the short arm of chromosome 4 of the Col and Ws accessions of *Arabidopsis thaliana*,

whereas this knob was absent in the related L*er* and C24 (Fransz et al., 1998). Previous investigations of knob accessions (Ws-2, Col) and knob-less accessions (Ler) (Fransz et al. 2000) revealed a reversed order of two BAC clones, T1J1 and T4B21, in the proximal region of chromosome arm 4S, suggesting a chromosomal rearrangement. It was suggested that this polymorphism resulted from an ancient paracentric inversion event. Later on in an analysis of crossover rates across chromosome 4 of a Col x Ler hybrid, Drouad et al. (2006) described a complete lack of crossovers in the proximal part of the 4S arm, which is also in support of heterozygosity for an inverted region.

In a separate extensive study on the nature and precise position of the inversion in Col and Ler, we mapped the inversion using FISH with BACs and smaller PCR products as probe on pachytene chromosomes, interphase nuclei and extended DNA fibres as hybridization targets (Fransz, unpublished work). Narrowing down the accuracy of the inversion breakpoints to less than 1 kb we are able to establish the precise breakpoints at nucleotide level accuracy, i.e., 1,612kb and 2,782kb from the NOR. The bioinformatics of the sequences around the breakpoint shed light on the putative ancestral transposon mechanisms leading to the rearrangement (Fransz, unpublished work).

In this paper we elaborate on cytogenetic consequences of this inversion in the case of a Col x Ler heterozygote. We use FISH of pooled BACs in the inverted region on DAPI stained spread pollen mother cells for establishing chromosome pairing. In addition, immunofluorescence microscopy of the synaptonemal complex proteins Asy1 and Zip1 on is applied to assess if the full SC is formed in the heterozygous inversion region (Armstrong et al., 2002; Chelysheva et al., 2005). We further designed primers around the proximal and distal breakpoints for a fast and easy PCR assay to demonstrate the inversion in 30 different accessions. By comparing the SNPs in the inversion and flanking DNA sequence regions of these accessions it now becomes possible to follow the introgression patterns between these accessions. A model for their underlying processes is discussed.

Material and methods

Plant material

We used the following accessions for isolating DNA samples and fixing young flower buds for microscopic preparations: Wassileskija (WS-0), WTC; Wassileskija (WS-2); Columbia, Col-0, MPI; C24, Gu-0 (Yo-0); Gückingen (Gu-0); Knox (knox-10); Knox (knox-18); Martuba (Mt-0); Pna (PNA10); SALK; Rmx (Rmx-A02); Rmx (Rmx-A180); RRS (RRS-10); RRS (RRS-07); Hannover (Ha-0); Ravensglas (Ragl-1); Enkheim (En-2); Siegen (Si-0); Mühlen (Mh-0); GIFU (Gifu-0); Toledo (Tol-0); Krotzenburg (Kro-0); Oberursel (Ob-0); Greenville (Gre-0); Turk Lake (Tul-0); Achkarren (Ak-1); Nw-0 (Neu-weilnau, Germany); Kashmir (Kas-2); Blackmount (Ba-1); Camberg (Ca-0); Landsberg erecta Ler-1 MPI (or Ler-0 WTC). Young

flower buds were collected in the morning, after fixed in fresh Carnoy's solution (Ethanol 96%: glacial acetic acid, 3:1) for one day, the flower buds were transferred the buds to 70% ethanol and store at 4 °C.

Slide preparation for FISH

We selected flower buds with diameter of 0.2-0.4 mm under the dissecting microscope, and rinse them two times in Milli-Q and once in 10 mM Na-citrate buffer (pH 4.5). Then the anthers were transferred to a standard pectolytic enzyme solution (0.3% of Cellulase RS (Yakult 203033, Yakult Pharmacecutical IND.CO, LTD, Tokyo, Japan), Pectolyase Y23 (Pectolyase from Aspergillus japonicus, Sigma Aldrich, St. Louis, MO, USA, P-3026) and Cytohelicase (Cytohelicase from Helix pomatia, Sigma Aldrich, St. Louis, MO, USA, C8274), 0.3 % final concentration each in the Na-citrate buffer for 3 hour at 37 ˚C. Anthers were washed in Milli-Q twice and left in ice water waiting further treatment. We transferred a single anther in a tiny amount of water on a clean slide and squeezed it with a fine needle to release the pollen mother cells. The cells were then spread and macerated by adding about 20 µL of acetic acid 45 – 60% (depending on the hardness of the tissue) for 1 minute on a 43 ˚C hotplate, followed by fixing the slide with drops of freshly prepared Carnoy's solution around and on top of the drop of cells in the acetic acid solution. The slides were then left to

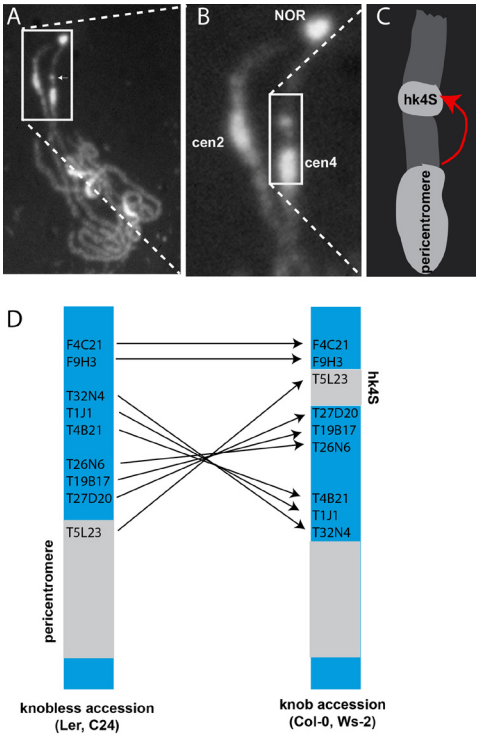


Figure 1. Cytogenetic reconstruction of the paracentric inversion in the short arm of chromosome 4. (A,B) DAPI-stained pachytene chromosomes of Ws-2 showing the bright heterochromatic knob hk4S (arrow). (C,D) Reconstruction of the inversion based on FISH analysis with BACs from the short arm of chromosome 4. The image in 1C corresponds to the dashed rectangular in 1A.

Table 1. List of BACs used in the FISH.

BAC	size in kb	total size
T14P8	94.5	637.3 kb
T10P11	113.6	
T5J8	98.5	
T4I9	98.9	
F4C21	136.3	
F9H3	95.5	
T27D20	80.4	389.2 kb
T19B17	105.8	
T26N6	99.4	
F4H6	103.6	
T19J18	43.2	267.1 kb
T4B21	89.9	
T1J1	53.8	
T32N4	80.2	

dry for few hours and the quality of the cell spreading checked under a phase contrast microscope equipped with 10x to 64x no-cover glass optics. Only slides with very well spread chromosomes with little or no cytoplasm were used for FISH and DAPI staining.

BAC isolation and Probe preparation

The following BACs and plasmid were used: T14P8, T10P11, T5J8, T4I9, F4C21, F9H3, T27D20, T19B17, T26N6, F4H6, T19J18, T4B21, T1J1, T32N4, T32A17, T3H13, F23J3 and pTa71 containing the 45S rDNA (Gerlach and Bedbrook 1979). BAC DNA isolation was done with the DNA extraction midi kit (Invitrogen detection technologies). All BAC clone DNAs were labeled with ARE DNA labeling kit (Invitrogen detection technologies).

Fluorescence *in situ* hybridization

The selected slides were pre-fixed with 1% formaldehyde in PBS, pH 6.8 at 37 ˚C overnight or at 65 ˚C for 30 minutes, followed by RNAse treatment (100 µg/mL DNase-free ribonuclease A stock solution (AppliChem, St. Louis, MO, USA) diluted as 1:100 in 2×SSC, pH 7) at 37 ˚C for 1 hour, then two times washed in 2×SSC and finally incubated in pepsin (Sigma Aldrich, St. Louis, MO, USA, the stock solution (100mg/mL in 10mM HCl) was diluted 1:100 in 10mM HCl). The slides were fixed again with 1% formaldehyde, washed in 2×SSC and dehydrated through an ethanol series (70%, 90%, and 96%) before air-drying. The probes were mixed with the hybridization mixture (50% formamide, 20% dextran sulfate), followed by thermal denaturation and stored on ice before put onto the slides. We added probe mixture on each slide and did the denaturation on a hot plate. Hybridization was carried out at 37 ˚C overnight. After hybridization, slides were washed at 42 ˚C three times in 50% formamide/-2×SSC, followed by two washes in 2×SSC. We then dehydrate the slides through an ethanol series and left them to air-dry again. The slides were counterstained with 12 µL DAPI in Vectashield (50 µL/mL) and examined under a Zeiss Axioplan 2 Imaging Photomicroscope, equipped with epi-fluorescence illumination and filter sets for DAPI, FITC, Cy3, Cy5, DEAC, and Cy3.5 fluorescence. The images were captured by a Photometrics Sensys 1305 x 1024-pixel CCD camera and processed with the Genus Image Analysis Workstation software (Applied Imaging). The raw images of DAPI and other fluorescent signals were captured in grey colour. When needed we further optimised brightness and contrast with Adobe Photoshop CS.

Slide preparation for immunofluorescence

Fresh, unfixed *Arabidopsis* flower buds were selected under the dissecting microscope on the basis of anther length and transferred to a slide in a petridish with moistened filter paper. The anthers of one flower bud were transferred to a digestion solution containing 4%

w/v Cytohelicase (Cytohelicase from Helix pomatia, Sigma Aldrich, St. Louis, MO, USA, C8274), 15% w/v sucrose in Milli-Q water) at 37 ˚C for 10 minutes. The anthers were then squashed with fine needles and left again in 10 µL digestion solution, after which 1% Lipsol™ (1% Lipsol in borate buffer pH 9.5) was added and mixed with a needle. Slides were kept on a hotplate at 37˚C for a few minutes, while adding small amounts of Lipsol to prevent them from drying, then few drops of 4% (para-)formaldehyde were dropped onto the cells, and the slide was left to dry in the fume hood.

Immuno detection of the primary antibodies

The primary Antibodies for ASY1 and ZYP1 polyclonal antibodies of *Arabidopsis thaliana* were obtained from Dr Liudmila Chelysheva (INRA, Versailles, France), and both were diluted 1:150 in EM blocking buffer (Armstrong et al. 2002; Higgins et al. 2005) were used as a dilution 1:150). The selected slides were washed three times in PBS and then transferred to a clean slide containing EM block (PBS-T-BSA, 1×PBS buffer, 1% w/v BSA, 0.1% v/v Triton), and kept in a humid box for 10 minutes at 20 °C. The primary antibody, diluted 1:150 in EM block, was put on the slide, and incubated in a humid box at 4 ˚C overnight or over the weekend. Then the slides were washed three times with PBS buffer. The secondary antibody (Rhodamine Red^{Tm}-X-conjugated* Af-finiPure Donkey Anti-Goat^{II} IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) or Fluorescein (FITC)-conjugated AffiniPure F (ab')2 Fragment Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:100 with EM block and put on the slide and left the slide in a humid box at 37 ˚C for 30 minutes. The slides were washed three times with PBS buffer, left to dry and then counterstained with 12 µL DAPI in Vectashield (50 µL/mL). The microscopic images were examined under a Zeiss Axioplan 2 imaging photomicroscope as explained above.

PCR amplification of genomic DNA

Genomic DNA was isolated with the "mini CTAB extraction DNA" method (method modified from Doyle 1990). PCR amplification reaction mixture, containing 0.4 U of Taq polymerase (Super Taq DNA Polymerases, Sphaero Q, TP05a), 10 times PCR buffer (including MgCl2), 5pM forward and reverse primers, 200uM dNTP's, 1ng genomic DNA, and Milli-Q water to make a final volume of 20 µL. The PCR reaction was set at 1 minute at 95 °C, followed by 35 cycles with 30 s of DNA denaturation at 94 oC, annealing at 58 °C for 30 seconds, and extension at 72 °C 1 minute, and final extension at 72 °C for 2 minutes. PCR was carried out in Bio-Rad iCycler Thermal Cycler. The following primers were used:
Primer 1. AACTCGATATTTCACAGGGC
Primer 4. GGAAGAGGCATCAAAAGGG
Primer 7. TCATCTACATGTCTTCCTCCAAC
Primer 8. AAACATATCCTACGAATACGC

Bioinformatics of the inversion region of selected accessions

Variant Call Format files were download from the 1001 genomes website (Cao et al., 2011; Schmitz et al., 2013; Wang et al., 2013) for 41 *Arabidopsis* accessions. For each accession a FASTA file was created by concatenating all Single Nucleotide Polymorphism (SNP) present. If for a given position no SNP was present, the nucleotide from the reference was assumed. This generates a multiple alignment file for each accession containing exclusively polymorphic positions (Lee et al., 2008). This FASTA file was fragmented in consecutive 50 Kbp segments and each of these were used to create a Jukes-Cantor distance matrix using FastTree2 software (Price et. al, 2010). Figure 5 shows the distance from each accession to the reference (A. thaliana v10) for each 50 Kbp fragment around the inversion. The lack of SNPs inside the inversion is a consequence of the introgression barrier created by the inversion.

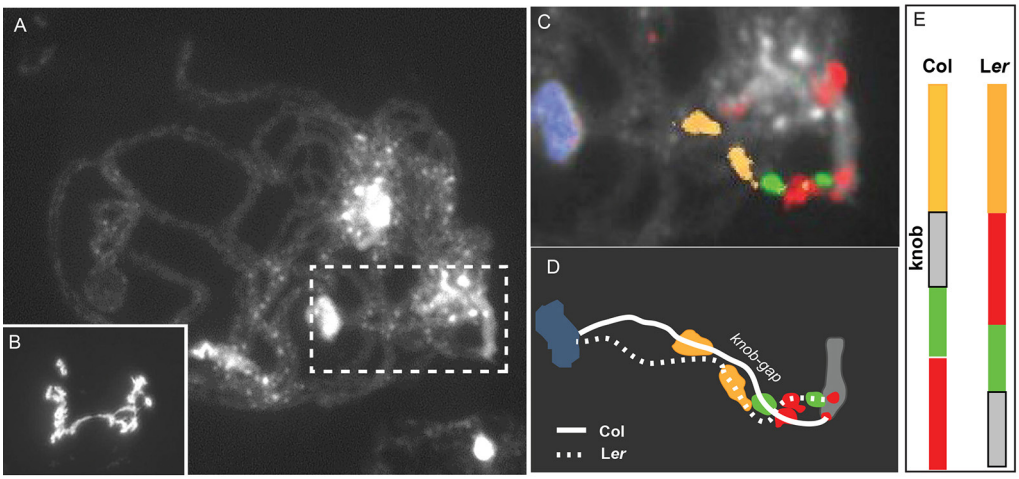
Results

Cytogenetic characterization of the inversion

The starting point for our study was a polymorphism of the heterochromatic knob, hk4S, on the short arm of chromosome 4 in the *Arabidopsis* Col-0 and Ws-2 (Fransz et al. 1998, 2000) and absent in Landsberg erecta, C24 and Cvi-0. A first series of FISH experiments with short arm chromosome 4 BACs on pachytene cells revealed an inverted arrangement of a chromosome region spanning the knob and proximal euchromatin regions (Figure 1A, Fransz et al., unpublished data). The data indicate that a paracentric inversion event has taken place in the proximal region of chromosome arm 4S that has moved the distal part of the pericentromere heterochromatin to interstitial euchromatin, thus creating the heterochromatic knob hk4S (Figure 1B).

To establish the position of the borders of the rearranged chromosome region we performed a second, more elaborative comparative FISH experiment with BACs, plasmids and PCR products as probes and compared their signals on interphase nuclei and extended DNA fibers on few knob and knobless accessions. This enabled us to position the breakpoints at 1612 ± 2 kb and 2780 ± 50 kb (Fransz et al., unpublished data).

Hybrids between knob and knobless accessions are heterozygous for the inversion and hence devoid of crossover recombinations in that region (Peters et al., 2001, Drouad et al. 2006; Koornneef, unpublished data). In this study we examined the chromosome behavior of the inversion during meiotic prophase I in Col-0 x Ler hybrids. To this end spread pollen mother cells were stained with DAPI, and examined under the microscope. The chromosomes displayed a regular alignment of the inversion region (Figure 2A). Inversion loops,



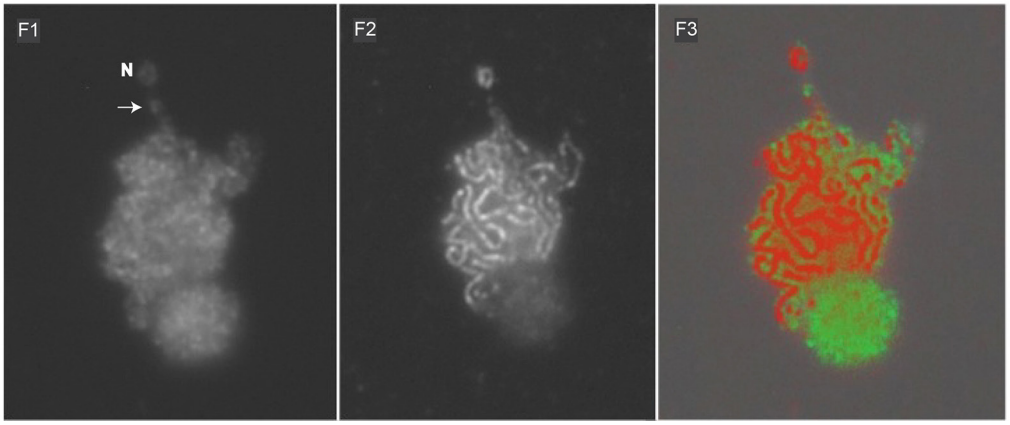


Figure 2. Cytogenetic analysis of chromosome 4 behavior in the heterozygote Col x Ler during meiotic recombination. (A, B) DAPI stained images of a pachytene cell showing paired homologues (A) and an anaphase I with a dicentric bridge (B). (C, D, E) FISH image (C) and reconstruction of FISH signals (D) based on the order of BAC probes along the linear chromosome (E). (F) Immunodetection of ASY1 (F1, green in F3) and ZYP1 (F2, red in F3) during pachytene. Arrows indicates proximal part of chromosome arm 4S.

however, were not observed, while only two anaphase I cells were found with a dicentric bridge (Figure 2B). We then hybridized the pachytene chromosomes with pooled BACs covering the distal and the proximal parts of the inversion along with a pool of BACs distal of the inversion (Table 1) together with a 45S rDNA repeat of the Nucleolar Organizer Region (NOR). The FISH signals showed a reversed order of the green and red foci in the inversion region, whereas the orange pool of BACs is collinear (Figure 2c-d), indicating the absence of

recombination between for the Col-0 and Ler homologues, which is typical for an inversed rearrangement. The results confirm an inversion event spanning the heterochromatic knob hk4S and the proximal euchromatin region of the short arm up to the border with pericentric heterochromatin.

According to the BAC-FISH on the F1 hybrid, Col x Ler, the two chromosomes did not pair at the inversion region. To investigate the recombination between the knob accession Col and the knobless accession Ler, we applied immuno-detection with two meiosis-related proteins Asy1 and Zip1. The Asy1 protein is associated with the chromosome axis of the synaptonemal complex (SC), while Zip1 is related to the central element of the SC. Zip 1 indicates synapsis between homologous chromosomes. In the F1 hybrid, nearly all chromosomes were paired and formed well-synapsed bivalents at pachytene stage, as fluorescent foci of Asy1 and Zip1 antibodies were overlapping (Figure 2F). However, in the proximal half of the short arm of chromosome 4 the two homologous chromosomes did not synapse at pachytene, as Zip1 fluorescence was lacking. The immunodetection results further confirm the absence of meiotic recombination in the inversion region.

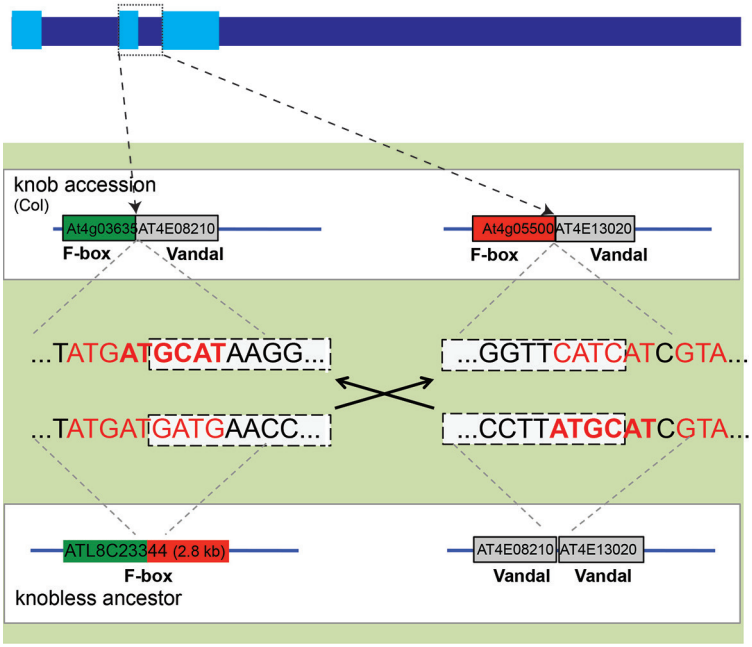


Figure 3. Sequence composition at the left and right breakpoints of the paracentric inversion. A pericentric *Vandal* transposon element (grey) in the knobless ancestor (bottom part) has inserted into an ATGATGATG fragment of a euchromatin F-box protein coding gene (red-green). The ATL8C23344 sequence is a *Ler* 2.8 kb fragment from the Cereon database that corresponds with the proximal (red) and distal (green) borders of the inversion in the knob accession (upper part).

Mapping the breakpoint of inversion region with PCR and karyotyping reveals many inversion accessions

By the FISH mapping approach we were able to position the distal and proximal inversion breakpoints at 2 kb and 50 kb precision, respectively. The use of sequenced Ler DNA fragments from the Cereon database (Monsanto Co., http://www.arabidopsis.org/browse/Cereon/index.jsp) enabled us to map the breakpoint of the inversion at base pair accuracy. The distal breakpoint maps at 1,612,602 bp and the proximal breakpoint at 2,782,611 bp (unpublished data). Moreover, the bioinformatics of the flanking regions revealed a Vandal transposon responsible for the chromosomal rearrangement by inserting into a F-box protein-coding gene (Figure 3). The results suggest transposition activity of a Vandal transposon element that inserted into an F-box protein-coding gene, causing a chromosomal arrangement that gave rise to the paracentric inversion.

The sequence information of the inversion enabled us to generate primers to identify the inversion breakpoints at nucleotide precision. This would allow us to find out if there are more *Arabidopsis* accessions that carry the inversion. We designed eight PCR primers of which four were used to distinguish between knob and knobless accessions, since these could be used as both forward and reverse primers for accession with and without the inversion (Fransz et al., unpublished data). The primer combinations 1+4, 4+8, 1+7, 7+8 successfully showed different bands between knob accessions (Col-0 and Ws-2) and knobless accessions (Ler and C24). These primer combinations were applied to other accessions that were assumed to carry the inversion based on limited SNP data (C. Toomajian, unpublished data). According to the PCR results, accessions Col, Gu-0, Knox-10, Mt-0, Pna-17, Rmx-A02, Rss-10, Yo-0, Ha-0, Ragl-1, En-2, Si-0, Mh-0, Gifu-0, Tol-0, Kro-0, Ob-0, Gre-0, Tul-0, Ak-1, Ba-1 and Ca-0 are knob accessions, while knox-18, Ler, Rmx-A180, Rss-7, Ws-0 and Kas-2 are knobless accessions (Figure 4).

To assess the presence of an inversion, we examined as to whether the putative knob accessions contain the heterochromatic knob hk4S. To this end we analyzed DAPI stained pachytene cells, which is the most appropriate stage to detect the heterochromatic knob. A bright DAPI positive structure in the short arm of chromosome 4 was easily identified in the accessions Col, Ws-2, Gu-0, Knox-10, Mt-0, Pna-10, Rmx-A02, Rrs-10, Yo-0, indicating the presence of the knob and thus the inversion (Figure 5A). No knob was observed in the accessions Knox-18, Ler, Rmx-A180, Rss-7, Ws-0, No-0, C24 (Figure 5B). The cytogenetic data confirm the presence or absence of the knob (or the inversion) shown by the PCR analysis (Table 2).

Recently the 1001 Genomes Project published the genomic sequence of many accessions (signal.salk.edu). We took advantage of this work to extent our knowledge and our conclusions with respect to identification and the phylogeny of knob accessions. Within the inversion region the inversion accessions showed a high level of sequence identity with Col-0, supporting the presence of the inversion. Outside the inversion the accessions differ from

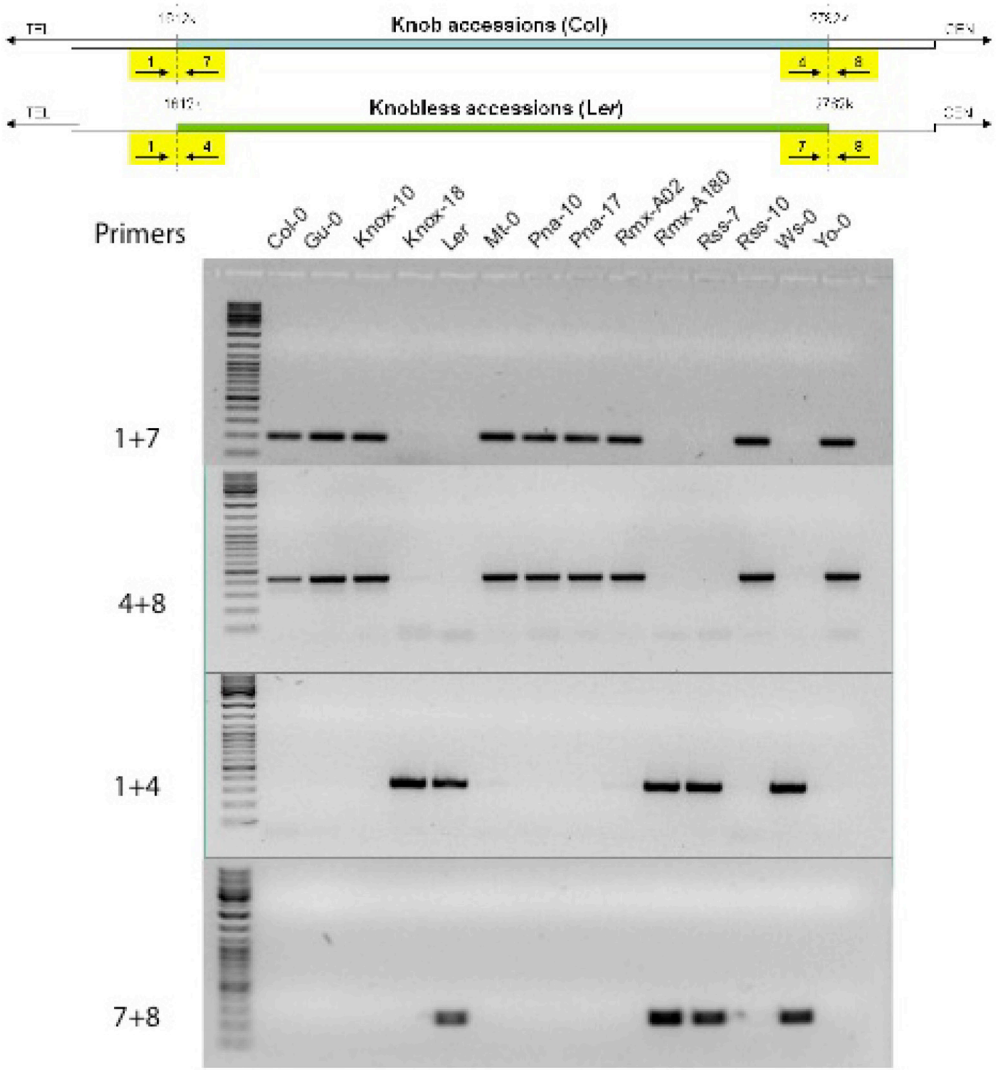


Figure 4. PCR analysis of the breakpoint sequences in *Arabidopsis* accessions. The primer combinations 1+7 and 4+8 provide bands only in knob accessions, whereas combinations 1+4 and 7+8 give bands only in knobless accessions.

each other and show many SNPs with the reference line Col-0 comparable to non-inversion accessions. We conclude that the inversion is transferred to other accessions worldwide most likely via introgression. The extreme low level of SNPs in the inversion region indicates that most sequence variation in the inversions region between knob accessions results from outcrossing with surrounding ecotypes rather than directly from mutations. We then applied the Introgression Browser (iBrowser), a novel bioinformatics method, which is based

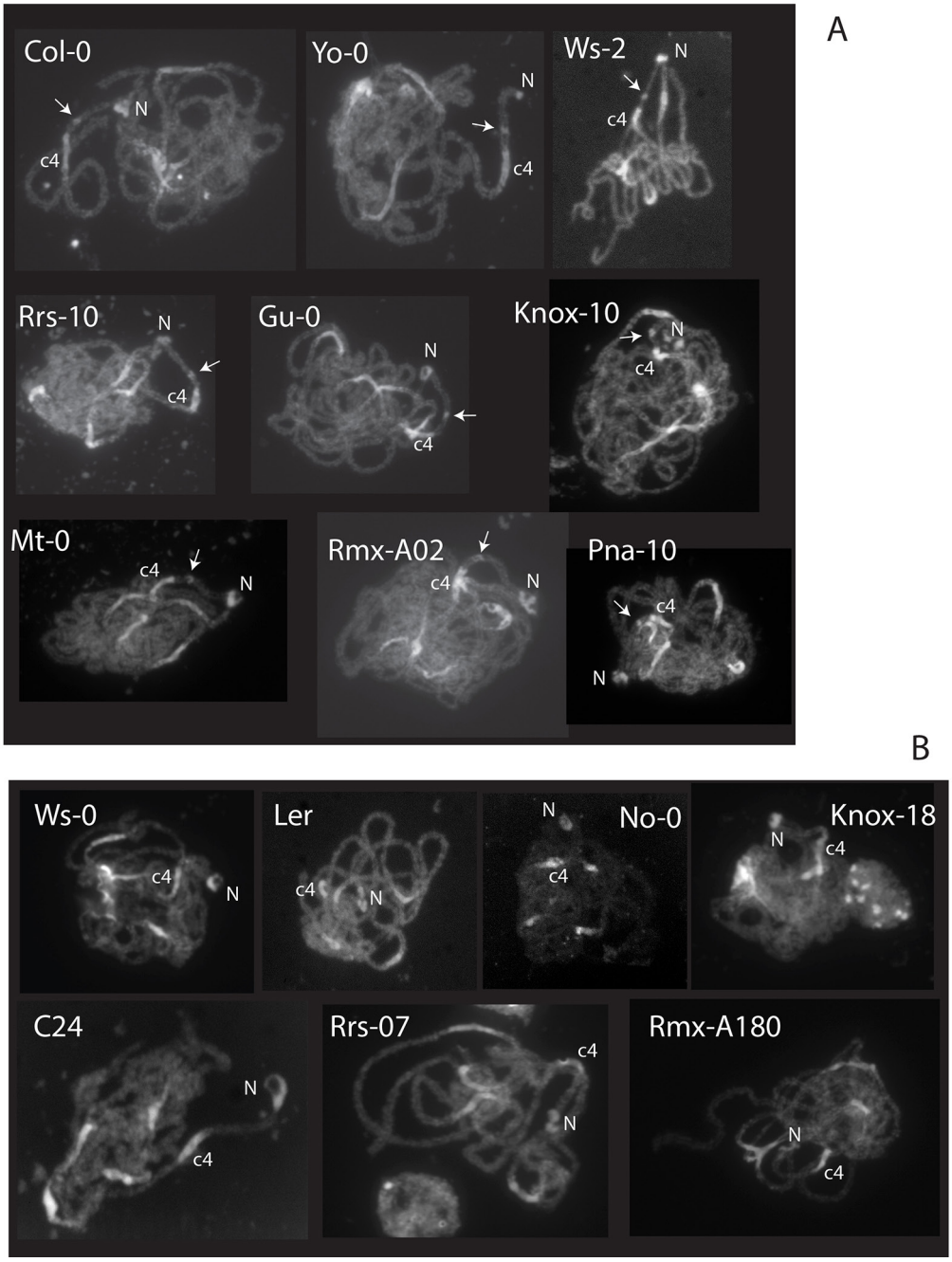


Figure 5. Microscopic analyses of the heterochromatic knob in pollen mother cells at pachytene of different accessions. DAPI staining shows the presence of the heterochromatin knob hk4S (arrows) in knob accessions whereas knobless accessions lack this heterochromatic structure in the short arm of chromosome 4.

Table 2. List of accessions examined for the presence or absence of the inversion and/or the heterochromatic knob.

knob accession	microscope	PCR	SALK database	distal recombination site	proximal recombination site
Ak-1	n.d.	Col	yes	1344	5247
Ba-1	n.d.	Col	yes	1540	5428
Ca-0	n.d.	Col	yes	1199	5634
Col-0	yes	Col	yes	0	18580
Do-0	n.d.	n.d.	yes	1588	5240
En-2	n.d.	n.d.	yes	1344	5247
Gifu-2	n.d.	Col	yes	1354	5166
Gre-0	n.d.	Col	yes	1354	5166
Gu-0	yes	Col	yes	1238	5596
Ha-0	n.d.	Col	yes	1530	5373
Knox-10	yes	Col	yes	1354	5166
Kro-0	n.d.	Col	yes	1344	5233
Mh-0	n.d.	n.d.	yes	1344	5299
Mt-0	yes	Col	no		
Nie1-2	n.d.	n.d.	yes	1391	5292
Nw-0	n.d.	n.d.	yes	1464	5427
Ob-0	n.d.	Col	yes	1344	4843
Pna-17	yes	Col	yes	1354	5166
Ragl-1	n.d.	Col	yes	1344	2822
Rmx-A02	yes	Col	no		
Rrs-10	yes	Col	yes	1354	5166
Si-0	n.d.	n.d.	yes	1464	4825
Star-8	n.d.	n.d.	yes	1344	5370
Tol-0	n.d.	Col	yes	1354	5166
Tul-0	n.d.	Col	yes	1354	5166
Ws-2	yes	Col	yes	1344	2822
Yo-0	yes	Col	yes	1354	5166

knobless accession	microscope	PCR	SALK database
Bak-2	n.d.	n.d.	no
Balan-1	n.d.	n.d.	no
C24	no	Ler	no
Cem-0-IP	n.d.	n.d.	no
Fei-0	n.d.	n.d.	no
Fun-0-IP	n.d.	n.d.	no
Gol-2	n.d.	n.d.	no
Hum-2-IP	n.d.	n.d.	no
ICE102	n.d.	n.d.	no
ICE104	n.d.	n.d.	no
ICE93	n.d.	n.d.	no
Kas-2	no	Ler	no
Knox-18	no	Ler	no
Ler-0	no	Ler	no
Li-7	n.d.	n.d.	no
No-0	no	n.d.	no
PNA3.40	n.d.	n.d.	no
Pra-6	n.d.	n.d.	no
Rmx-A180	no	n.d.	no
Rss-7	no	Ler	no
Set-1	n.d.	n.d.	no
TeuWa1-2	n.d.	n.d.	no
Tuscha9	n.d.	n.d.	no
Vie-0	n.d.	n.d.	no
Ws-0	no	Ler	no

on SNP information and allows examining introgressions at high resolution (Alves Aflitos et al., unpublished results). The visualization of SNP patterns enables to recognize boundaries of introgression regions and haplotype structures. Here we present a heatmap covering 8 Mbp of chromosome 4, showing phylogenetic distances between 42 accessions (with or without the inversion). The SNP data are derived from the Salk database. Knob accessions are easily recognized by the absence of SNPs in the inversion region (1612 kb - 2782 kb), indicating close relationship for this region. Outside the inversion region the high degree of similarity among inversion accessions remains until the number of SNPs increases dramatically, and hence increasing phylogenetic distance, here indicated by the appearance of grey boxes. This increase in SNPs (phylogenetic distance) points at the boundary of the introgressed region that carries the inversion and marks a recombination event between

accessions. Some inversion haplotypes share the same recombination position suggesting that they are derived from the same recombination. Most inversion accessions also share the same pericentromere haplotype (2800 kb - 4800 kb), since it contains very few SNPs. An exception is formed by Ws-2 and Ragl-1. These accessions have introgression site in the pericentric heterochromatin of the short arm close to the inversion breakpoint, which points at a very rare recombination event. It also indicates that the two accessions share the same ancestor (progenitor) for the introgression haplotype. The data show that accessions can be grouped according to common introgression recombination sites. Based on the phylogenetic distance in the inversion region we have now identified more than 50 inversion accessions, of which 37 are from Europe with Germany having 13 inversion accessions. Interestingly, nine of the German accessions are located in the area around Frankfurt, suggesting a putative origin of the ancestral inversion accession in this region.

Discussion

This study presents the identification of a paracentric inversion that contains a heterochromatic knob hk4S in the short arm of chromosome 4 of *Arabidopsis*. Although interstitial heterochromatic knobs have been well documented in eukaryotic chromosomes (Lima-de-Faria, 1976; Guerra, 2000), little is known about their origin. Several explanations for interstitial knobs have been proposed: (i) spontaneous or transposon driven transfer of distal satellite repeats between spatially neighboring inter-chromosomal regions (Bennett, 1982; Zhong et al., 1998; Szinay et al. 2010a), (ii) DNA methylation and heterochromatinization (Golyshev et al., 2008; Soppe et al., 2002; Zhang et al., 2008), (iii) heterochromatinization in sex chromosomes (Kejnovsky et al., 2009) and (iv) (retro)transposon repeat accumulation (Ananiev et al., 1998; Lamb et al., 2007). This study is the first to claiming that a knob can evolve from a chromosomal rearrangement event having one of the breakpoints within a heterochromatic region.

The presence of the inversion has genetic consequences for the chromosomal region, since the inverted orientation of the chromosomal segments in the inversion impedes synapsis and genetic recombination. Indeed, a cold spot of meiotic crossovers has been reported in the proximal part of chromosome arm 4S (Schmidt et al., 1995, Drouaud et al., 2006). Our cytogenetic data confirm the recombination conflict in the inversion region. Even outside the inversion the homologues appear to have problems in proper alignment. As a consequence no recombination is possible in the inversion region in plants that are heterozygous for the inversion and the inversion region will inherit as a single locus and becomes fixed in inversion accessions.

We have mapped the breakpoint positions of a 1.17 Mb paracentric inversion in *Arabidopsis thaliana* with base pair accuracy. The inversion is composed of a 680 kb segment of the pericentromeric heterochromatin, which gives rise to the heterochromatic knob, and

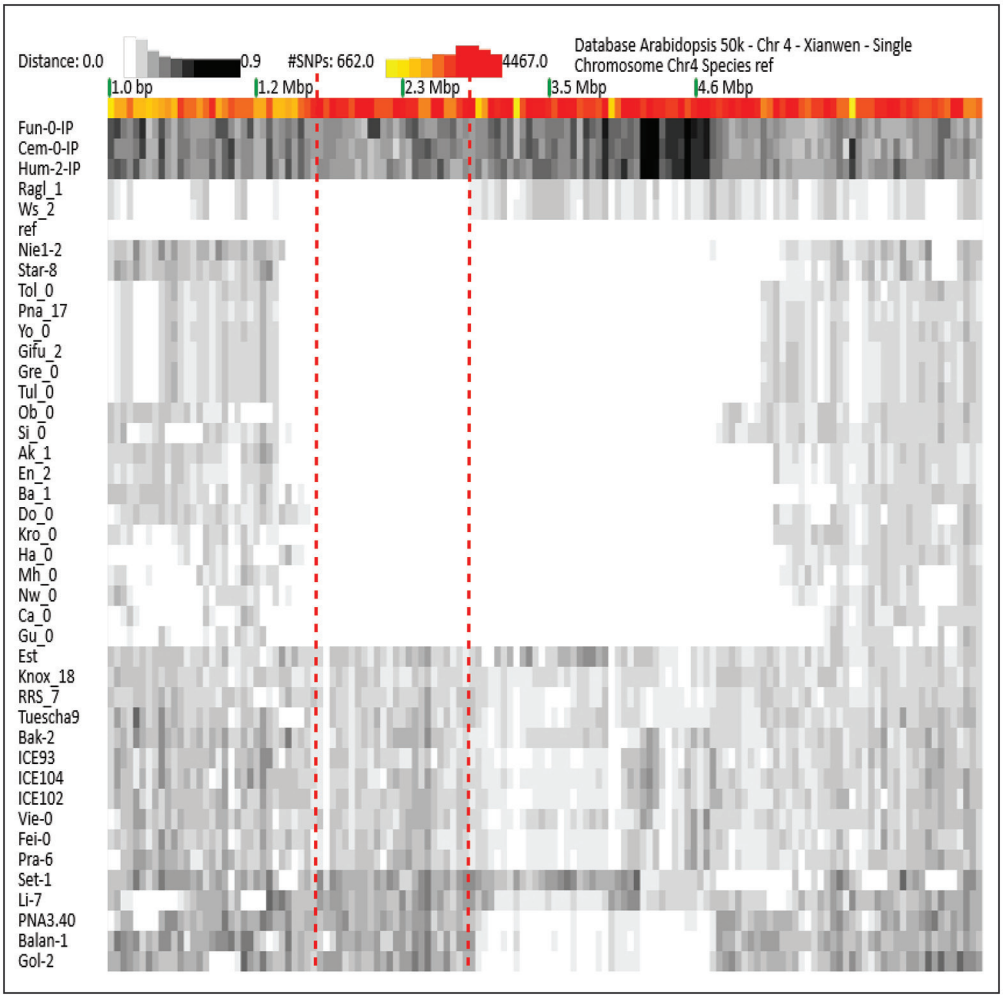


Figure 6. Fragment of chromosome 4 between position 1 and 8.5 Mbp from *Arabidopsis thaliana* for 42 accession from the Salk database. Each column represents a 50 Kbp window. Each row represents one accession. Each block represents the Jukes-Cantor sequence distance between each accession and the reference *Arabidopsis thaliana* v10 (Columbia). Grey scale is the sequence distance between each block and the reference. Orange blocks are the number of SNPs in each column used to calculate the distance. Color values are described in the top of the graph. White blocks are regions with low dis-tance to the reference, consequence of the lack of crossover inside the region. The red dashed lines represent the left and right border of the inversion.

a 490 kb euchromatic region. The entire inversion region contains 144 genes of which the majority is located in the euchromatin (McCombie et al., 2000). The presence of Vandal elements at both breakpoints suggests the involvement of Vandal transposition activity in the inversion event. It is known that transposable elements (TEs) can generate chromosomal inversions via homologous recombinations between TE copies, known as TE-mediated ectopic recombination or nonallelic homologous recombination (Petes and Hill, 1988). In

Drosophila it has been demonstrated that the transposon Galileo generates natural chromosomal inversion via recombination between ectopic sites (Delprat et al., 2009). In the flowering plant *Anthirrinum majus* the inversion of a chromosomal segment was generated by the activity of the transposon element Tam3 (Robbins et al., 1989). The molecular mechanism involves the physical association between donor and recipient sites in such a way that one end of the transposon remains attached to its original site. The result of this aberrant transposition is a chromosomal inversion. We propose that a similar mechanism occurred on chromosome arm 4S in *Arabidopsis*, involving the mutator-like transposon Vandal, of which there are several fragments at both inversion breakpoints.

We have identified over 50 accessions carrying the inversion, distributed over the world (Europe, America and Asia). Considering the high number of inversion accessions in Germany the ancestral inversion accession might originate in Central Europe and then spread to other parts of Europe. A comparison of the inversion sequence between the knob accessions suggests that the inversion event may have taken place within the last 10.000 years (C. Toomajian, pers. comm.). This period coincides with the colonization of Europe by *Arabidopsis* from Asia and Mediterranean refugia after the last glaciation (Sharbel et al., 2000). The presence of inversion lines in America is possibly due to recent migration activities of settlers across the Atlantic Ocean (Jorgensen and Mauricio, 2004, Peter and Slatkin, 2014). The sequence of the inversion region in knob accessions is similar to each other and to the reference accession Col-0, whereas outside the inversion the number of SNPs becomes comparable to knobless accessions. The low SNP frequency in the inversion suggests that in the past 10,000 years spontaneous mutation did not occur frequently which indicates that most polymorphisms in the entire genome is due to outcrossing with other accessions. This is remarkable, since *Arabidopsis* is a selfing species. Despite the selfing nature of *Arabidopsis* this species has managed to distribute a chromosomal inversion worldwide to other populations via introgressive hybridization. If the an inversion carries locally favorable alleles, the plant will benefit of having the inversion haplotype. Due to suppressing recombination between loci in the inversion region, the inversion can spread (Kirkpatrick & Barton, 2006). The presence of some knob and knobless accessions in the same accession background (e.g. Ws-0 and Ws-2 or Rrs-10 and Rrs-7) may point at a process of introgressive hybridization that is still continuing. The presence of the inversion in many accessions provides a unique system to follow the distribution of a haplotype among *Arabidopsis* populations. Moreover, it enables to assess recent recombination events between the ancestors of current *Arabidopsis* accessions and to understand the dynamics of haplotypes in *Arabidopsis*.

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Chapter 6

General Discussion

In my thesis I have described two cytogenetic phenomena that play a significant role in plant breeding practices: 1) aberrant meiosis leading to unbalanced gametes and hence to aneuploid offspring and 2) heterozygosity for an inversion that is associated with linkage drag. As to the former, high incidence of aneuploidy is a serious problem in cauliflower (Brassica oleracea var. Botrytus) cultivation and breeding as plants with extra chromosomes in their cell complement display an aberrant phenotype often with a very small curd or no curd at all. As such aberrant phenotypes can sometime amount more than 10% of an offspring family, it is obviously that aneuploidy in cauliflower generates an enormous economic loss (Chable et al., 2008). Aberrant morphology of aneuploid plants is very common and was reported for many species (e.g., Epstein et al. 1988; Gearhart et al. 1987; Huettel et al. 2008). As shown in Chapter 4, the cause for aneuploidy in cauliflower is the desynaptic nature of meiosis, but the genetic basis of this erratic process of chiasma formation is still not elucidated. Chable et al. (2009) hypothesized that aneuploid formation is induced by epigenetic changes, but their results were not clearly unequivocal. Moreover, additional experiments also suggest effects of environmental changes and stress on the incidence of aneuploid offspring.

The second phenomenon that I mentioned above is the manifestation of inversions and other structural chromosome rearrangements that can be revealed in a comparative study of related accessions or species. The consequences of such rearrangements have been described in Chapter 5, using the model species *Arabidopsis thaliana* for studying cytogenetic and genomic consequences in an inversion heterozygote. The outcome of this study is directly appropriate for plant breeders in their effort to circumvent linkage drag in introgressive hybridization programs.

Is there a relation between structural chromosome mutations, like inversions, and aberrant meiosis? One may argue that this is not likely the case. After all inversion heterozygotes can be diagnosed clearly by the observation of dicentric bridges and acentric fragments at anaphase I / II, in the case crossovers take place in the inversion loop and interstitial region between loop and centromere. We did observe very few of such bridges in anaphase I of the Col X Ler inversion heterozygote *Arabidopsis* (Chapter 5, Figure 2B), but never observed any such bridges in cauliflower meiosis. Translocation heterozygotes do have their own landmark in meiosis: they often exhibit quadrivalents at diakinesis, and when neighbor centromeres cosegregate they will lead to unbalanced gametes and hence gives rise to sterile spores. Although such associations were observed in cauliflower they seem the exception rather than the rule and so are not considered a plausible clarification for the high univalent numbers. Large duplications and deletions are also most unlikely as they occur only rarely in plants. The next aberration that we observed in diakinesis / metaphase I complements are the interconnections between bivalents, a phenomenon that sometimes might be interpreted erratically as chiasmate bonds. Although a clear explanation is lacking, their incidence is pretty common in interspecific hybrids, inbred lines and desynaptic plants (Orellana and Giraldez, 1983; Cermeño et al. 1985) and may have resulted from erratic replication of tandem repeats at premeiotic interphase, disturbances or out-of-synchrony of different

meiotic processes or delayed tapetal development leading to improper detachment of the non-homologues at their tandem repeats (Chapter 4). It is not likely that bivalent interconnections have ensued from structural rearrangements or from the desynaptic nature of meiosis that we diagnosed in cauliflower and not in the cauliflower mutant of *Arabidopsis*.

The most significant impairment of cauliflower meiosis that I mentioned is desynapsis featuring regular chromosome pairing at pachytene and numerous univalents at diakinesis. Similar meiotic disturbances were observed in previous research on *Rosa rugosa* (Klášterská and Natarajan, 1974), who supposed that their disturbance occurs at the diffuse stage, shortly after pachytene; at the time the synaptonemal complex disintegrates (Ekberg et al. 1968; Owens and Molder 1971; Storlazzi et al. 2008; Bressa et al. 2002). While others have suggested the failure of proper chiasma formation in desynaptic plants happens during the diffuse diplotene stage (Caryl et al. 2003; Jauhar and Singh, 1969), we have some evidence that in the case of cauliflower this is an earlier event. We observed that the ZMM-protein MLH1, that initially loads onto chromosomes during prophase I, localizes onto bivalent interconnections and found that desynapsis does not lead to a reduction in the total numbers of crossovers in the cell. The crossovers rather localize onto other chromosomes. The genetic basis for this incomplete chiasma formation is most problematic as the phenotype shows variable penetrance (Pagliarini et al. 2011; Jackson et al. 2002) and is often prone to environmental factors (Chable et al., 2009; unpublished own observations).

Inversions and linkage drag

Heterozygosity for a paracentric inversion is a big issue for plant breeders as meiotic recombination in the inversion region does not allow homologues to pair, and if they pair, they form a loop in which a single crossover will lead to a dicentric bridges and fragments at anaphase I, and sometimes also at anaphase II if a second crossover takes place between inversion loop and centromere. In the case of a pericentric inversion (across the centromere) duplication – deletion chromosomes will be formed instead that will give rise to 50% infertile gametes. The ultimate consequence of an inversion, either paracentric or pericentric, is that the parental allele combinations within the inversion cannot be unlinked. For breeders it means that a desirable allele (or hemizygous gene) for a desirable trait like disease resistance and a neighbor gene responsible for an unfavorable trait cannot be broken by crossover, the phenomenon that we know as linkage drag.

A solution to this problem is not easy to find. First of all it is worth the effort to explore all possible resources of wild relatives of the crop that contains the desirable gene / allele the breeders want to introduce in their crop. As genes can be polymorphic so are inversions and other chromosome rearrangements, and it therefore makes sense to check large numbers of wild relatives for the presence or absence of this chromosome rearrangement. A second solution may be feasible in those cases that the paracentric inversion is relatively large, i.e., it occupies most of the chromosome arm. In that case the normal and inversion chromosomes

may likely form an inversion loop. As said, a single crossover in such a loop will lead to an anaphase bridge and hence gets lost, as the formed gamete is not viable. But in the situation with two crossovers in the loop involving the same two nonsister chromatids (double 2 strand crossovers) the inverted region will become shorter, possibly breaking the linkage between desired and unwanted genes. Such rare 2-strand double crossovers were reported in various cytogenetic studies like barley (Ekberg 1974), but high levels of crossover interference in that region may aggravate the change for such double crossovers very much. If (genetic or chiasma) interference could be circumvented by upregulating crossover numbers by increasing the numbers of interference-independent crossovers (Crismani et al., 2012), the incidence of double 2-strand crossovers might be increased.

Studies on structural chromosome aberrations have benefitted enormously from DNA sequencing and bioinformatics (Laufs et al. 1999, Nacry et al. 1998, Tsujimoto et al. 1999). As was shown in Chapter 5, comparative genomics of the region with and without the inversion in *Arabidopsis* revealed precise mapping of the inversion breakpoints. If sequencing data are not available, FISH with BACs and smaller vectors might position such breakpoints, but their accuracy will never be better than in the range of kilo base pairs. Once the breakpoints are established, primers can be designed for demonstrating their positions by PCR. With the PCR test, it is possible to apply on larger number of accessions to determine the presence of a knob in the chromosome arm 4S. This molecular test is much faster than any other genetic, cytogenetic and genomics method and hence suitable for screening larger numbers of genotypes and accessions, and even wild relatives meant for possible donors in introgressive hybridization programs.

Methodological aspects of the cytogenetic experiments

Chromosome identification allows researchers to identify chromosome segments, individual chromosomes and whole chromosome sets in a cell complement, and so establish numerical and structural chromosome changes for karyotype evolution or aneuploid diagnosis. However, in many species, the morphology of their chromosomes is quite similar, which hampers karyotype analysis without extra tools like banding and FISH technologies. In plants more than in mammals chromosomes contain genomic dispersed repeats that are homogenized across all chromosomes (Schmidt and HeslopHarrison 1998), which made it difficult to apply strategies as Chromosome *in situ* suppression hybridization (CISS), which were developed for mammal chromosome identification (Hultén et al. 1991; Jauch et al. 1990). The alternative of CISS hybridization for chromosome identification in plant species is repetitive painting and BAC painting, which uses genomic information to select chromosome specific sequences as probe. Several plant species were successfully subjected to this method for karyotyping, as maize, tomato, rice and barley (Kato et al.2004; Chang et al. 2008; Ohmido and Fukui 1995; Busch et al. 1995).

The repeat painting of the cauliflower chromosomes of Chapter 2 has shown that the use of only five different repeats is sufficient for distinguishing individual chromosomes in the cell complement, to identify them in the karyotype and diagnose the extra chromosomes in trisomic individuals. With repeat painting, we could identify trisomies for all nine chromosomes in cauliflower. The method is straightforward and relatively simple, but has few major drawbacks. Firstly, three of the repeats are part of the pericentromere, and most likely different members of LTR retrotransposons. They are present in some chromosomes but absent in others. Proper interpretation of the multicolour fluorescent signals requires that different colors can be switched on and off in the imaging software as they partly overlap. This is less convenient than the case in which all signals are located on different chromosome regions, and do not overlap. Secondly, comparisons of the multicolor repeat banding patterns with those of other *B. oleracea* chromosome studies have shown quite some repeat polymorphisms (Howell et al. 2002; Lim et al. 2005, 2007; Xiong and Pires 2011), and so the repeat painting for the time being is limited to the material that we now studied. For other cauliflower cultivars, and especially for *B. oleracea* cultivars and varieties genetically more distant, a new series of repeat experiments would have to be carried out for chromosome identification. The third drawback is that repeat banding is limited to the genetic inert part of the genome: that of the NOR, centromeres and pericentromeres. As such, small deletions in gene rich euchromatin areas will go unnoticed by repeat painting studies. In addition, repeats can be no starting point for further genetic and genomic studies, while single copy FISH does.

To meet in to the objections of the repeat painting we developed an adapted version of the cross-species painting with BACs. Here, *Arabidopsis* BACs are hybridized to other species and crops of the *Brassica* family. The technology originally developed by Lysak et al (2001, 2003, 2005, 2006) was based on repeat poor BACs on all five *Arabidopsis* chromosomes and was used for chromosome structure (Lysak et al. 2007), interphase organization (Fransz et al. 2000; 2002), evolutionary relations in the *Brassica* family. Cross species FISH was applied in many plant species for the study of structural chromosome rearrangements, as potato, pepper and tomato, *Arabidopsis* and *Brassica* species, sorghum and maize (Peters et al. 2012; Tang et al. 2008; Lysak et al. 2003, 2006, 2007; Amarillo and Bass 2007). Here, we showed the technology to elucidate the nature and breakpoints of the inversion in the Columbia accession of *Arabidopsis* and for painting the cauliflower chromosomes to establish multicolor FISH karyotypes and to identify extra chromosomes in aneuploid plants. In my chapter 3 I gave only one example of cross-species BAC FISH on cauliflower, based on pools from the chromosomes 1, 2 and 3 that we used most in our study were available at the moment I started this study. Now with all five chromosome BAC libraries at hand, many more painting combinations can be made to identify other parts of the cauliflower chromosomes faster and unambiguously. Our cross-species BAC FISH painting result suggested each *Arabidopsis* BAC sequences had two or three even four copies on *B. oleracea* genome, due to an ancient genome triplication in the *Brassica* genome (see below). The advantages of

this technology are obvious: one can elucidate the nature of structural and numerical aberrations as well as clarify specific chromosome region related information with corresponding genetic and physical maps. On the flip side of the coin, cross-species BAC FISH painting is labor intensive. This drawback can be reduced by substituting BAC DNAs by pools of PCR products, specific for each chromosome region of interest (Lamb et al. 2007).

Cytogenetics moves to cytogenomics

The genomes of *Brassica* species have duplicated during evolution, while some segment of the Brassica genome were reshuffled by inversions and translocations, leading to the so called chromosomal diploidization (Ziolkowski et al. 2006). Most of this research was based on the genome comparison between *Arabidopsis* and *Brassica* (Kowalski et al. 1994; Osborn et al. 1997; Bowers et al. 2003), but showing nice insights into the evolutionary relationships between the two species. Lysak et al. (2005, 2007) applied cross species BAC FISH with segments of the *Arabidopsis* genome to *Brassica* species, indicating the *Arabidopsis* segments are usually triplicated on *Brassica* genome. Later the whole-genome comparison between *A. thaliana* and *Brassica* species revealed the complete extent of this whole-genome triplication between the two genomes (Wang J. et al. 2011; Wang X. et al. 2011), and is also confirmed by our MUMmerplot analysis in Chapter 3.

Chapter 5 goes one step further: BAC FISH demonstrates the position of a pericentric inversion and identifies its breakpoints. Meiotic analysis of the inversion heterozygote evidenced the lack of crossovers in that region, which genetically explains the lack of recombinants in offspring. Furthermore, it explains in terms of comparative genomics that the inverted region of the accessions with the heterochromatic knob cannot recombine, and therefore shows less SNP variation as is seen in non-knob accessions. We have also seen that the DNA sequence information around the proximal and distal breakpoints of the rearrangements allows us to create PCR primers with which larger numbers of genotypes / accessions can be traced for presence / absence of the inversion.

Developments in the near future

The experiments that I described and the discussions I gave on the background, all leave open ends. I showed two methods to distinguish chromosomes in a crop and to identify extra chromosomes in aneuploid organisms. Either have its advantages and drawbacks, but they are above all time consuming and not suitable for high throughput analyses. In an effort to demonstrate aneuploidy in larger populations, Henry et al. (2006) presented a novel technology for whole genome karyotyping, by quantitative fluorescence-polymerase chain reaction (QF-PCR) on heterozygous aneuploid *Arabidopsis*. The method determines deviating dosages (allelic ratios) for different markers on chromosomes. This method can in theory readily be applied in a crop such as *B. oleracea*. An additional aspect that pops up is the

question what the effects are of aneuploidy for a certain chromosome, and to what extend does the extra chromosome deregulates the balance of gene dosage on a genomic scale, and hence do change the phenotype of that plant. The second challenge for aneuploidy in cauliflower is elucidating the molecular mechanism for the desynaptic meiosis. Which genes are involved in the disturbance of the crossover machinery and how can one best select for this trait in a breeding program? Even more so, are there sex-dependent differences in the rate of univalents formation? In other words, can we expect that overall crossover rates in female desynaptic meiosis of cauliflower are higher or even lower than in the male meiosis (cf. Drouaud et al. (2007) and de Vicente and Tanksley (1991) for differences in recombination rates between male and female meiosis).

A second major leap in future cytogenetics can be expected from its integration with comparative genomics (Aflitos et al., 2014). I already mentioned that BAC FISH painting can shed light on specific chromosome areas, that supplemented with marker-assisted breeding, can are the genomic regions that are crucial for introgressive hybridization. Knowledge of such regions now effectively points at homeologous regions and can help the introgression of the desirable traits into crops (Szinay et al. 2010).

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Summaries

in English, Dutch, Chinese

Summary

In this thesis, I describe several cytogenetic tools to study numerical and structural chromosome aberrations of cauliflower (*Brassica oleracea* var. *botrytus*) and *Arabidopsis thaliana*. Such large-scale changes in the genome are indisputably important for controlling gene balance, expression and regulation, and so can change the phenotype. Moreover, chromosome changes, in particular polyploidy, inversions and translocations play a significant role in evolution. Here I focus on some of such changes, their origin and implications for genetic, genomic and plant breeding research. The technologies that I used for my study are advanced karyotype analysis based on Fluorescent *in situ* Hybridization (FISH) with repetitive and single copy sequences as probes, chromosome identification in aneuploids, analysis of meiosis that give rise to high rates of aneuploid gametes, and characterization of an inversion using PCR, chromosome painting and immunofluorescence of meiotic proteins.

The General Introduction in chapter 1 deals with a historical overview of numerical and structural chromosome variants, and especially their occurrence and significance in plant species. I also explain why and how chromosomes are sorted in clear chromosome set portraits displaying how they are ordered and identified on the basis of their morphology. The FISH technology plays a central role in my thesis and I therefore explain extensively the different types and their applications. The contribution of genomics in cytogenetics is described and finally I give examples how cytogenetics can play a role in solving crossing problems in plant breeding programs.

Chapter 2 and 3 presents two strategies to identify individual chromosomes in cell complements of cauliflower. The ultimate goal is to identify the chromosomes in aneuploid plants with aberrant phenotype, which cause huge economic loss for breeders. In chapter 2, I describe the results of a chromosome painting based on FISH with five different repetitive sequences as probes. The multicolour images thus obtained enables us to produce karyotypes in which all chromosomes could be identified and the extra chromosome (arms) in aneuploid individuals determined. In other words, the repeat painting now allows the correlate different trisomies and specific aberration phenotype, like small, early flowering or ugly curd shapes. However, comparison of our repeat FISH karyotypes results with those in related studies revealed clear polymorphisms in different varieties of *B. oleracea*.

To overcome the drawbacks of repeat polymorphisms I worked out an alternative chromosome painting strategy, based on cross-species BAC painting with BACs from *Arabidopsis* on cauliflower chromosomes. In chapter 3 I showed the first results of this FISH technology. As BAC clones in cross-species FISH have less repetitive sequences in common with the target chromosomes on which they hybridize, the technique is outstanding in displaying the position of single copy DNA sequences on the cauliflower chromosomes. Taking into account that the genome of cauliflower is triplicated compared to *Arabidopsis* we used genomic information in MUMmer plots comparing *Arabidopsis thaliana* and *Brassica oleracea*. The final result of the multicolor FISH that was developed nicely demonstrated unique

chromosome bands with which all cauliflower chromosomes could be identified. Compared with repetitive FISH painting, cross-species BAC FISH painting is likely more ubiquitous and can more easily be applied on other *Brassica* species.

Chapter 4 describes the experiments on male meiosis of cauliflower aiming at elucidating the cause of meiotic disturbances responsible for aneuploid gametes, and so aneuploid progeny with aberrant phenotypes. To this end I compare male meiosis of three cauliflower lines, which produce different rates of aneuploid progeny. Microscopic observations revealed that chromosome pairing, but that chiasma formation is disturbed or incomplete leading to variable rates of univalents at diakinesis and metaphase I, and consequently to unbalanced gametes and aneuploid offspring. We also observed large numbers of interconnection between chromosomes and bivalents that are composed or tandem repetitive sequences, but is not clear if these connections are responsible for aneuploid gametes.

In chapter 5, a cytogenetic and genomic study on a paracentric inversion on chromosome arm 4s of *Arabidopsis thaliana* is described. A small heterochromatin knob in the middle of the short arm euchromatin, which was observed in Col and few other *Arabidopsis* accession, while absent in L*er* and most other accessions, was found resulting from this inversion. To further investigate the inversion region I used immunofluorescence of synaptonemal complex proteins and BAC FISH painting for the meiotic behavior of the inverted region in a Col x Ler hybrid. The two chromosome regions in the inversion were found not to synapse, in spite of the incidental anaphase bridges that were observed at anaphase I. Further BAC FISH painting and sequence comparison around the borders of the inversion revealed the breakpoints of the inversion at nucleotide precision. PCR tests with primers around both breakpoints allowed assessment of the presence of the inversion in another 30 accessions, and these results were compared with microscopic analysis of pachytene complements for demonstrating the heterochromatic knob. SNPs were found absent in accessions with the inversion due to absence of crossover recombination in that region. We demonstrate such SNP free regions in several accessions using the recently developed iBrowser bioinformatics tool. The software not only confirmed the SNP free inversion region but also demonstrated that accessions can have different flanking SNP free sequencing due to rare ancestral crossover sites.

In my final chapter I discuss the results obtained especially in the context of what breeders may help in their breeding programs: identifying chromosomes in aneuploid progeny, elucidating the desynaptic meiotic disturbances leading to unbalances spores and the consequences of inversions and other structural rearrangements for linkage drag in introgressive hybridization programs.

Samenvatting

In mijn proefschrift beschrijf ik verschillende cytogenetische methoden om numerieke en structurele chromosoomafwijkingen te bestuderen in bloemkool (*Brassica oleracea* var. *botrytus*) en *Arabidopsis thaliana*. Zulke grootschalige veranderingen in het genoom zijn onmiskenbaar belangrijk voor het controleren van genbalans, genexpressive en genregulatie, en speelt daarom een grote rol op het fenotype. Verder spelen chromosoomveranderingen, en met name polyploïdie, inversies en translocaties een belangrijke rol in de evolutie. Bij mij ligt de nadruk op sommige van die veranderingen, hun oorsprong en betekenis voor genetisch, genomisch en plantenveredelingsonderzoek. De technieken die ik daarbij gebruikte zijn geavanceerde karyotype analyse, gebaseerd op Fluorescente in situ Hybridisatie (FISH) met repeterende en unieke DNA sequenties as probes, chromosoomidentificatie in aneuploïden, analyse van de meiose die leidt tot hoge frequenties aneuploïde gameten, en karakterisering van een inversie d.m.v. PCR, chromosoomschildering en immunofluorescentie van meiose-eiwitten.

De Algemene Inleiding in hoofdstuk 1 geeft een historisch overzicht van numerieke en structurele chromosoomvarianten, met nadruk hun voorkomen en betekenis in plantensoorten. Ik leg ook uit hoe chromosomen gesorteerd worden in overzichtelijke portretten van complete chromosoomsets, waarin ieder individueel chromosoom kan worden onderscheiden op basis van hun morfologie. FISH technologie speelt een centrale rol in mijn proefschrift en ik leg daarom de verschillende typen en hun toepassingen uit. De bijdrage van genomica in de cytogenetica wordt ook beschreven aan de hand van een aantal voorbeelden waarin de rol voor het oplossen van kruisingsproblemen in de plantenveredeling worden uitgewerkt.

Hoofdstukken 2 en 3 gaan over twee methoden om individuele chromosomen van complete delende cel-sets van bloemkool te identificeren. Het uiteindelijke doel daarbij is om chromosomen in aneuploïde planten met een afwijkende fenotype, die voor de kwekers een enorme economische schadepost betekenen, te identificeren. In hoofdstuk 2 beschrijf ik de resultaten van een chromosoomschildering gebaseerd op FISH technologie met repetitieve DNA sequenties als probes. De veelkleurenplaatjes die aldus worden verkregen stellen ons in staat om karyotypen te maken die waarin alle chromosomen konden worden geïdentificeerd en de extra chromosoom(-armen) in aneuploïde individuen kunnen worden vastgesteld. Met andere woorden, de kleuren met repetitieve sequenties stelt ons nu in staat om verschillende trisomen te associëren met specifieke afwijkende fenotypen, zoals kleine vroegbloeiende bloeiende bloemkolen, of lelijke afwijkende koolvormen. De vergelijking van onze resultaten met deze techniek met de resultaten in verwante publicaties laten echter zien dat er duidelijke polymorfieën tussen verschillende variëteiten van B. oleracea bestaan. Om aan dit nadeel tegemoet te komen heb ik een alternatieve techniek uitgewerkt waarin DNA van de verwante Arabidopsis thaliana als probe gehybridiseerd wordt op de chromosoompreparaten van de bloemkool. Deze zogenaamde “cross-species BAC FISH painting”

leg ik verder uit in hoofdstuk 3 aan de hand van een aantal voorbeelden. Omdat het probe DNA in dit soort experimenten weinig of geen last heeft van hybridisatie van de repetitieve DNA die Arabidopsis en bloemkool gemeen hebben, is de techniek uitermate geschikt om unieke DNA sequenties op de bloemkoolchromosomen aan te tonen. Het probleem is echter dat het bloemkoolgenoom in feite getripliceerd is, dus dat het drie keer voorkomt vergeleken met dat van Arabidopsis. Door gebruik te maken van een bioinformatica applicatie (MUMmer) kunnen we nu zien welke sequenties van Arabidopsis voorkomen op de chromosomen van bloemkool, en zodoende kunnen we met specifieke DNA sequenties van Arabidopsis een veelkleurige FISH bandering op de bloemkoolchromosomen maken waarin alle chromosomen op basis van hun kleurenbandjes kunnen worden onderscheiden. Vergeleken met de repeat bandering zoals ik in hoofdstuk 2 beschreef is deze "cross-species BAC FISH" veel algemener toepasbaar en veel reproduceerbaarder.

In hoofdstuk 4 beschrijf ik de experimenten betreffende de mannelijke meiose in bloemkool. Doel is om de oorzaak van meiotische onregelmatigheden aan het licht te brengen die de oorzaak zijn voor aneuploïde gameten, en dus voor aneuploïden nakomelingen met afwijkend fenotype. Ik heb hiertoe drie bloemkool genotypen met elkaar vergeleken, die in verschillende mate afwijkend nakomelingschap produceert. We noemen die lijnen "slecht", middelmatig" en "goed". Microscopisch onderzoek toonde aan dat de chromosoomparing normaal is, maar dat vorming van chiasmata verstoord of onvolledig is wat weer leidt tot een variabel aantal univalenten, en bijgevolg tot meer of minder univalenten in de diakinese en metafase I stadia, en dus ongebalanceerde gameten en aneuploid nakomelingschap. Ik beschrijf ook de grote aantallen verbindingen tussen de chromosomen en bivalenten in die cellen, waarvan kon worden aangetoond dat die uit tandem repetitieve sequenties bestaan. Het is evenwel niet duidelijk of die verbindingen verantwoordelijk zijn voor (deel van) de aneuploïde gameten.

In hoofdstuk 5 wordt een cytogenetisch en genoomstudie van een paracentrische inversie op chromosoomarm 4S van Arabidopsis thaliana beschreven. Een kleine heterochromatische verdikking in het midden van het korte arm euchromatine kon worden waargenomen in de Columbia accessie (Col) van *Arabidopsis*, terwijl een dergelijke structuur in Landsberg *erecta* (L*er*) ontbrak. Deze verdikking blijkt nu het gevolg te zijn van een inversie waarbij een stukje van het heterochromatin uit de pericentromeer in het midden van de korte arm terecht kwam. Voor verder onderzoek gebruikte ik immunofluorescentie van synaptonemale complex-eiwitten voor de bestudering van het meiotisch gedrag in en rond de inversie van een Col x L*er* hybride. De twee chromosoomgebieden van de inversie blijken nu geen synapsis te vormen, in tegenstelling tot de anafase I bruggen die we incidenteel in het materiaal hadden waargenomen. Door middel van FISH chromosoomkleuring en vergelijkend DNA sequentieonderzoek was het mogelijk om de breukpunten van de inversie tot nucleotideprecisie aan te tonen. Door nu gebruik te maken van PCR met primers rond deze breukpunten aan weerszijde van de inversie konden we nu de aanwezigheid van de inversie op grote schaal bij 30 accessies onderzoeken. Deze resultaten werden vervolgens vergeleken met de micros-

copische analyse van pachytene cellen voor het aantonen van de heterochromatische verdikking. SNPs waren grotendeels afwezig in accessies met de inversie. Dit is het gevolg van ontbreken van crossover recombinatie in dat gebied. Dergelijke SNP-vrije gebieden in verschillende accessies konden nu vergeleken worden met een recent ontwikkelde iBrowser bioinformatica applicatie. Deze software kon niet alleen de SNP vrije inversiegebieden aantonen maar liet ook overduidelijk zien accessies verschillende flankerende SNP vrije gebieden kunnen hebben die de posities van zeer zeldzame crossovers in het verleden blootstellen.

In mijn laatste hoofdstuk discussieer ik over de verkregen resultaten in de context van wat plantenveredelaars er aan kunnen hebben. Dus het identificeren van chromosomen in aneuploïde nakomelingen, het aan het licht brengen van desynaptische verstoringen tijdens de meiose die leidt tot ongebalanceerde gameten en daarmee tot aneuploïde nakomelingen. Ook ga ik in op de consequenties van inversies en andere structurele herrangschikkingen voor het optreden van absolute koppeling tussen gewenste en ongewenste eigenschappen in introgressieve hybridisatieprogramma's.

中文摘要

本论文主要描述几种细胞遗传学技术，这些技术于研究菜花(Brassica oleracea var. botrytus)和拟南芥(Arabidopsis thaliana)染色体的数量和结构变异。在基因组中这样巨大的变化对于控制基因平衡，基因的表达和调控是非常重要的，甚至可以改变表现型。特别是在多倍体中，倒位、易位等染色体上的改变，在生物进化中起到了重要的作用。本文主要研究染色体改变的起源和对遗传，以及基因组学和植物育种的影响。本文使用的细胞遗传学技术是染色体核型分析包括：荧光原位杂交(以重复序列和单拷贝序列为探针)，非整倍体染色体鉴定，分析可产生大量非整倍体配子的减数分裂过程，以及使用聚合酶链反应鉴定染色体倒位，萤光免疫检验法检测减数分裂蛋白质。

第一章综述了染色体的数量和结构变异，特别是植物中发生的染色体变异。同时论述了通过染色体形态鉴定染色体的原因和方法。本论文主要使用荧光原位杂交的技术，因此本章将着重论述荧光原位杂交的种类和应用。本章还论述了基因组学对细胞遗传学研究的贡献，以及细胞遗传学在解决杂交育种方面发挥的作用。

第二章和第三章描述了两种鉴定菜花染色体的方法，以鉴定表型异常的非整倍体的染色体。第二章描述了以重复序列为探针的荧光原位杂交，我们使用五种荧光素标记五个重复序列，根据荧光原位杂交的结果，我们可以鉴定染色体以及识别非整倍体中额外增加的染色体(染色体片段)。以重复序列为探针的荧光原位杂交可以让特定的三体与特定异常表型相关联。将我们的荧光原位杂交结果与相关研究比较，我们发现不同品种的甘蓝存在明显的多态性。

为了克服重复序列在不同品种甘蓝基因组的多态性质疑问题，我使用了跨物种荧光原位杂交，此方法标记拟南芥基因序列为探针与菜花染色体杂交。第三章阐述了以这种原位杂交鉴定染色体。此原位杂交使用的BAC序列和将与之杂交的目标染色体间有较少的共同重复序列，因此，此方法可用于在菜花染色体上定位单拷贝序列。我们使用MUMmer plots对菜花基因组和拟南芥基因组进行了基因对比，结果显示菜花基因组是拟南芥基因组的三倍复制，我们根据基因对比结果筛选几组拟南芥BAC pool标记为探针，跨物种荧光原位杂交可以鉴定菜花基因组中的所有染色体。与以重复序列为探针的荧光原位杂交相比，跨物种荧光原位杂交更容易应用于甘蓝的染色体鉴定。

第四章描述了对菜花花粉母细胞减数分裂行为的研究，以探讨粉母细胞减数分裂异常导致非整倍体配子形成以及非整倍体后代产生的原因。我比较了三组产生不同比率非整倍体后代菜花样本的花粉母细胞减数分裂行为。通过显微镜观察花粉母细胞减数分裂显示，染色体虽然成功配对但减数分裂期交叉形成却被干扰，导致减数分裂终变期和减分一中期不同比率单价体的产生，最终形成非整倍体配子和非整倍体后代。我们也观察到大量的串联现象发生在染色体或四分体之间，造成串联现象原因是重复序列之间互连的，但尚不清楚是否这现象引发非整倍体配子的产生。

第五章阐述的是对拟南芥四号染色体短臂臂内倒位的细胞遗传学和基因组学研究。

小异旋钮在短臂常染色质，其观察到胶原和其他一些拟南芥加入的中间，而没有在LER和大多数其他的加入，结果发现从该反演得到的。为了进一步探讨反型区我用联会复合体的蛋白质和BAC FISH绘画免疫在一处山坳倒地区的减数分裂行为X LER混合。之前的研究在Col和其他几个拟南芥品系的常染色质短臂上发现一个小异染色质染色体球节，但此球节并没有在Ler和绝大部分的拟南芥品系染色体中发现，倒位被认为是此球节的形成原因。我使用了免疫荧光检

测联会复合体蛋白和荧光原位杂交以进一步探讨倒位区域在Col x Ler杂合体中的粉母细胞减数分裂行为。在Col x Ler杂合体的倒位区域内，没有发现联会现象，尽管在Col x Ler杂合体减分一后期观察到了后期桥。进一步的荧光原位杂交和对倒位区域的序列对比确定了倒位在染色体上发生的断裂点。使用以断裂点周围的序列设计的引物进行PCR检测可以确定是否在四号染色体短臂出现到位现象，我们检测了30个拟南芥品系，PCR结果与显微镜观察减分一粗线期结果相对比，以确定PCR检测的正确性。在到位发生的拟南芥品系中，我们没有在到位区域发现SNPs，由于该区域没有交叉重组。我们使用生物信息软件在很多品系中发现这种无SNP区域。此软件不仅确认了到位区域没有SNP，而且也在品系中发现一些侧翼无SNP序列，这些序列的形成原因可能是罕见的祖先交换位点。

第六章着重讨论了本论文的实验结果，特别是在育种方面的作用：鉴定非整倍体后代的染色体，阐明减数分裂过程中的desynaptic干扰导致了非整倍体配子的形成，以及倒置等结构重排引发的连锁累赘。

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to:	Xianwen Ji
Date:	5 December 2014
Group:	Genetics
University:	Wageningen University & Research Centre, The Netherlands

1) **Start-up phase**	date
◊ First presentation of your project	
Chromosome painting technologies for the analyses of chromosome organisation and meiotic behaviour in models and crop species of the Brassicaceae family	Nov 01, 2011
◊ Writing or rewriting a project proposal	
Chromosome painting technologies for the analyses of chromosome organisation and meiotic behaviour in models and crop species of the Brassicaceae family	Apr 201
◊ Writing a review or book chapter	
◊ MSc courses	
Genetic analysis tools and concepts (plant genetic part)	Dec 200
◊ Laboratory use of isotopes	
Subtotal Start-up Phase	9.5 credits*

2) **Scientific Exposure**	date
◊ EPS PhD student days	
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, Amsterdam University	Nov 30, 2012
◊ EPS theme symposia	
EPS theme 4: 'Genome Plasticity', Radboud University	Dec 11, 2009
EPS theme 4: 'Genome Plasticity', Wageningen University	Dec 10, 2010
EPS theme 4: 'Genome Plasticity', Wageningen University	Dec 09, 2011
EPS theme 4: 'Genome Plasticity', Radboud University	Dec 07, 2012
EPS theme 4: 'Genome Plasticity', Wageningen University	Dec 13, 2013
◊ NWO Lunteren days and other National Platforms	

NWO-ALW meeting 'Experimental Plant Sciences', Lunteren — Apr 19-20, 2010
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren — Apr 22-23, 2013

◊ **Seminars (series), workshops and symposia**

Mini symposium: How to write a world-class paper — Oct 26, 2010
ExPectationS Day (EPS Career Day), Wageningen University — Nov 19, 2010
ExPectationS Day (EPS Career Day), Wageningen University — Nov 18, 2011
EPS Mini-symposium 'Plant Breeding in the genomics era' — Nov 25, 2011
Summer School 'Natural Variation of Plants', Wageningen — Aug 21-24, 2012
DNase I hypersensitity: a signature of open chromatin and its application in plant genome and biology research, Amsterdam — Oct 02, 2012
Eric Schranz: Whole genome duplications as drivers of evolutionary innovations and radiations? — Nov 21, 2013

◊ **Seminar plus**

◊ **International symposia and congresses**

European Retreat of PhD Students In Experimental Plant Sciences, Cologne, Germany — Apr 15-17, 2010
European Retreat of PhD Students In Experimental Plant Sciences, Paris, France — Jul 05-08, 2011
International Chromosome Conference, Manchester, UK — Aug 29-Sep 02, 2011
Plant Genome Evolution, Amsterdam, NL — Sep 04-06, 2011
European Retreat of PhD Students In Experimental Plant Sciences, Norwich, UK — Aug 15-17, 2012

◊ **Presentations**

European Retreat of PhD Students In Experimental Plant Sciences, Cologne, Germany (poster) — Apr 15-17, 2010
European Retreat of PhD Students In Experimental Plant Sciences, Paris, France (poster) — Jul 05-08, 2011
International Chromosome Conference, Manchester, UK (poster) — Aug 29-Sep 02, 2011
European Retreat of PhD Students In Experimental Plant Sciences, Norwich, UK (poster) — Aug 15-17, 2012
Summer School 'Natural Variation of Plants', Wageningen UR, NL (oral) — Aug 21-24, 2012

EPS theme 4: 'Genome Plasticity', Wageningen UR, NL (oral)	Dec 07,2012
ALW meeting 'Experimental Plant Sciences', Lunteren, NL (oral)	Apr 22-23, 2013
◊ IAB interview	
Meeting with a member of the International Advisory Board of EPS	Nov 15, 2012
◊ Excursions	
Subtotal Scientific Exposure	18.6 credits*

3) **In-Depth Studies**	date
◊ **EPS courses or other PhD courses**	
Practical course: 'Bioinformatics: A User's Approach'	Aug 30-Sep 3, 2012
Course:'Mixed model based genetic analysis in GenStat: from QTL mapping and association mapping to genomic prediction'	Sep 2-4, 2013
course: 'Basic Statistics'	Dec 13-15,20,21, 2012
◊ **Journal club**	
◊ **Individual research training**	
lab visit to IAN (France)	Mar 15-16, 2012
lab visit to Birmingham	Aug 13, 2012
Subtotal In-Depth Studies	4.8 credits*

4) **Personal development**	date
◊ **Skill training courses**	
Information Literacy for PhD including Endnote introduction	Apr 27-28, 2010
Advanced course Guide to Scientific Artwork	May 10-11, 2010
Career Perspectives	Sep19, 26 & Oct 03, 10,17
PhD Competence Assessment	Mar 22 & 28, 2011
Course: Techniques for writing and presenting a scientific paper	Apr 24-27, 2012
◊ **Organisation of PhD students day, course or conference**	
◊ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	4.2 credits*

TOTAL NUMBER OF CREDIT POINTS*	**37.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

* A credit represents a normative study load of 28 hours of study.

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