Meiotic Aberrations Leading To Aneuploidy In Cauliflower (*Brassica Oleracea* L. var. *Botrytis*)

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Abstract

Aneuploid cauliflower plants (*Brassica oleracea* L. var. *botrytis*) display abnormal curd phenotypes causing serious commercial problems in offspring populations. Despite extensive breeding efforts, selection of genotypes producing euploid gametes remains unsuccessful due to unknown genetic and environmental factors. To reveal the origin of aneuploid gametes, we analyzed chromosome pairing, chiasma formation and chromosome segregation in pollen mother cells of selected cauliflower genotypes. To this end we compared different genotypes exhibiting Low with < 5%, Moderate with 5-10% and High with > 10% aberrant offspring. Microscopic observations revealed regular chromosome pairing at pachytene. However, cells at diakinesis and metaphase I showed variable numbers of univalents, suggesting that chiasma formation during meiotic prophase is incomplete or disrupted and results in a partial desynaptic phenotype. Cells at anaphase I – telophase II exhibited various degrees of unbalanced chromosome numbers explaining the aneuploid offspring. Immunofluorescence probed with an MLH1 antibody demonstrated fluorescent foci in all genotypes, but their lower numbers do not correspond to the putative sites of chiasmata. Interchromosomal connections between chromosomes and bivalents are common at diakinesis and metaphase I, and they contain centromeric and 45S rDNA tandem repeats, but such threads seemed not to affect proper disjoin of the half bivalents at anaphase I. Moreover, male meiosis in the arabidopsis *APETALA1/CAULIFLOWER* double mutant with the typical cauliflower phenotype did show interchromosomal connections, but there were no indications for partial desynapsis. We now hypothesize that the occurrence of desynapsis in cauliflower is a developmental out-of-phase phenomenon partially or completely controlled by genes involved in flower and curd development.

Introduction

Polyploid and aneuploids plants are known to produce trisomics and other numerical variants in their self-fertilized or backcrossed offspring. The most common explanation is that plants with unequal chromosome numbers may have unbalanced chromosome segregation during meiotic divisions and so lead to aneuploid gametes. In addition, extra copies of chromosomes in the germ line may also result from incidental mitotic or meiotic non-disjunctions, but these events are sporadic. An exception is cauliflower (*Brassica oleracea* L. var. *botrytis*) in which considerable numbers of aneuploids offspring are formed from diploid progenitors. Such plants with characteristic small and irregularly shaped curds cause considerable economic loss to growers and breeders. Despite extensive breeding efforts, selection of genotypes producing euploid gametes is unsuccessful as both genetic, epigenetic, and environmental factors affect regular meiosis with balanced gametes (Chable et al. 2008; 2009).

A well-known cause of unbalanced chromosomes in the germline is caused by incomplete or absent pairing of homologous chromosomes, known as asynapsis. Examples of genes involved in the upstream part of meiotic recombination machinery, such as *ASY1* and *SPO11* (e.g., Ross et al. 1997, Wei and Zhang 2010), are known to completely disrupt early steps in the pairing initiation and crossover processes and normally lead to complete loss of control of meiosis and gamete degeneration. The second group is that of desynapsis mutants, which are characterized by regular chromosome pairing or synapsis at early

meiosis (i.e., the formation of a functional tripartite synaptonemal complex), but fail to complete crossover formation, resulting in less or no chiasmata (Ross et al. 1997). Other meiotic aberrations that can also lead to the unbalanced segregation of chromosomes, include merotelic kinetochore attachment in which a kinetochore attaches to microtubules 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). More 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 comprehensively in reviews of *Arabidopsis thaliana* (Castellano and Sablowski 2008, Peirson et al. 1997, Yang et al. 1999, Zamariola et al. 2014), *Brassica napus* (Souza and Pagliarini 1996), wheat (Huskins and Hearne 1933), maize (Beadle 1937; Beadleg 1933; Caetano-Pereira et al. 1995), tomato (Soost, 1951) and others. Unbalanced gametes may give rise to aneuploid offspring, as was demonstrated for various crops and model species including *Arabidopsis* (Grelon et al. 2003), wheat (Griffiths et al. 2006) and maize (Carlson et al. 2007).

In this study, we studied as to whether meiotic aberrations might be causal to the occurrence of aneuploids among cauliflower offspring. We focus on chromosome processes during male meiosis in cauliflower, which may lead to unbalanced gametes and hence, to aneuploid offspring. We suggest desynapsis is the most likely mechanism leading to less chiasmata, leading to the subsequent formation of univalents at diakinesis / metaphase I and so to unbalanced chromosome segregation at later stages, and finally to aneuploid gametes. We also noticed abundant interchromosomal connections and asked the question whether this relates to decreased number of chiasmata and more univalents. In addition, we speculated that allelic variants of genes involved in curd formation itself can, as a side effect, induce erratic meiosis. We therefore investigated meiotic stages in the pollen mother cells of the *APETALA1/CAULIFLOWER* double mutant of *Arabidopsis thaliana* (Bowman et al. 1993; Smyth 1995) with the typical cauliflower like curd morphology.

Material And Methods

We selected three genotypes of cauliflower (*B. oleracea* L. var. *botrytis*) that differ in proportion of aneuploid individuals in their offspring. Aneuploid individuals among offspring were identified by their typical aberrant phenotypes and samples of these plants were measured by flowcytometry, confirming trisomy. The so-called Low genotype produces less than 5% aneuploids; the Moderate genotype has 5-10% aneuploids and the High genotype more than 10% aneuploids. The *APETALA1/CAULIFLOWER* double mutant of *Arabidopsis thaliana* with a cauliflower like curd was obtained from Dr. Kerstin Kaufmann (Bioscience, Plant Research International, WUR, Wageningen (Bowman et al. 1993)).

Repetitive DNA sequence for specific chromosome painting

The plasmid pTa71 containing the 45S rDNA repeat (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 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' -CAGGAAAACCTGGGATCACCTGATTAAAT-3'

Slide preparation

Young flower buds of cauliflower and *APETALA1/ CAULIFLOWER* double mutant of *Arabidopsis thaliana* were fixed in freshly prepared ethanol: acetic acid, 3:1) at 20 °C and after one day transferred to ethanol 70% for longer storage at 4 °C. Anthers were dissected from the flower buds, washed in purified water two times and in 10 mM Na-citrate buffer (pH 4.5), and then mildly digested in a pectolytic enzyme mix, containing cellulase RS (Yakult Pharmaceutical IND.CO, LTD, Tokyo, Japan, Yakult 203033), pectolyase Y23 (from *Aspergillus japonicus*, Sigma Aldrich, St. Louis, MO, USA, P-3026) and cytohelicase (from *Helix pomatia*, Sigma Aldrich, St. Louis, MO, USA, C8274) in the Na-citrate buffer at a 0.3% final concentration for each. The flower buds were macerated for 3 hours at 37 °C in the enzyme solution. 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 ethanol: acetic acid (3:1) fixative was dropped around and on top of the cell suspension, after which slides were left to dry for few minutes. Slides were checked under the phase contrast microscope for quality control (Kantama et al. 2017).

Fluorescence in situ hybridization

We labelled the 45S rDNA probe directly with Di-ethylaminocoumarin-5-dUTP (DEAC, Perkin Elmer Life Sciences, Boston, MA, REF: NEL-455001EA), the Centbr1 probe was labelled indirectly using the Biotin-Nick-translation for Cy5 detection (Roche Applied Science, REF: 11745816910) and for the Centbr2 probe we labelled indirectly with the Dig-Nick-translation for FITC detection (Roche Applied Science, REF: 11745824910) as described previously (Kato et al. 2004). Probes of three repetitive sequences were hybridized to various slides of the same genotype. Cell spreads were pre-treated 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).

Ten µL of each DNA probes was added to 10 µL hybridization mixture containing 50% formamide, 20% dextran sulphate, 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 an 80 °C hot plate for 3 minutes denaturation. 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 detection 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 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 airdried slides were counterstained with 12 µL of a 300 ng/mL DAPI (4', 6-diamidino-2-phenylindole, Sigma Aldrich, St. Louis, MO, USA, D-1388) in Vectashield® antifade mounting medium (Vector Laboratories) 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, Cy5 and DEAC. The images were processed with Genus Image Analysis Workstation software (Applied Imaging) and selected images were captured by a Photometrics Sensys monochrome 1305 3 1024-pixel CCD camera. Different fluorescent signals were captured and combined in Adobe Photoshop by using overlapping layers, gradient colour mapping and screen blend mode (Kantama et al. 2017).

Immunodetection of MLH1

For the detection of the mismatch repair protein MLH1, class I crossover protein, we performed immunofluorescence microscopy with primary antibodies for this protein on air-dried acetic spread pollen mother cells at diakinesis. The MLH1 polyclonal antibody of *Arabidopsis thaliana* was obtained from Dr Liudmila Chelysheva (INRA, Versailles, France). We followed her protocol (Chelysheva et al. 2010) with few adaptations. To this end we heated 10 mM Na-citrate buffer (pH 6.0) in a microwave at 450 W for 45 seconds until boiling, submerged the slides in the boiling solution and cooked this in the microwave at 450 W for 45 seconds. 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-TBSA (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 300 ng/mL DAPI in Vectashield and covered with a 24×50-glass cover slide. Microscopy and image processing followed the description above. We pseudo-coloured the DAPI image in red for better contrast with the green MLH1 foci.

Statistics of the numerical data

We selected mostly diakinesis / metaphase I cells and quantified the number of rod and ring bivalents, univalent pairs, chiasma bonds and chromosomes involved in inter-connections. Basic statistical analyses were performed in Microsoft Excel, GenStat v. 16.2 (VSN International Ltd, <http://www.vsnl.co.uk/software/genstat>) and JASP v. 0.14.1 (<https://jasp-stats.org/getting-started-with-jasp/>).

Results

Meiotic aberrations in the three genotypes

The basis of our analysis is the comparison of morphological and quantitative meiotic characteristics between three cauliflower genotypes that differ in the rates of offspring with aberrant phenotype. The so-called Low phenotype produces less than 5% aberrant offspring, the Moderate phenotype between 5 and 10% and the High phenotype more than 10%. For each genotype we studied around 50 DAPI stained pollen mother cells (PMCs), between pachytene – anaphase II, of which the majority were at diakinesis (more than 50 PMCs per genotype were observed).

Figures 1 (a, b) show representative examples of meiotic prophase cells at pachytene. Chromosomes at this stage in all genotypes are fully paired and display the typical pattern of brightly fluorescing pericentromeres, that are often tightly clumped forming a dense synizetic knot (Moens 1964), often considered the result of an acetic fixation artefact (Armstrong et al. 2001). The distal parts of the chromosome arms are the less condensed euchromatin with various smaller chromomeres. Although in some nuclei smaller chromosome regions are not paired, it is quite rare in our observation, we consider chromosome behaviour at this stage synaptic. Incidentally we distinguished in the three genotypes associations between non-homologous centromeres or pericentromeres (Figure 1a).

At diakinesis, chromosomes are highly condensed and chiasmata bonds, resulting from diplotene chiasmata are clearly distinguishable (Figure 2a-e). For the interpretation of the bivalent configurations, since chromosome arms can have only one chiasma, so two adjacent chiasmata in one arm will be interpreted as a single chiasma. Without at least one chiasma homologues are not attached and the two chromosomes remain as a univalent pair (Figure 2c). With only one chiasma between homologues, bivalents attain the shape of a cross or that of a rod, depending on the position of the chiasma (Figure 2a). With two chiasmata, a bivalent forms different shapes depending on proximal position, *i.e.*, close to centromere or distal position, close to the chromosome end (Figure 2b). Most cells contained ring and rod

bivalents and univalent pairs (Figure 2 d, e). In the Low genotype, univalents are rare and occur in only 10.5% of the cells. In the Moderate and High genotypes univalents are much more frequent (39% and 42.3% respectively). Interchromosomal connections between the homologous and non-homologous chromosomes are seen in all genotypes, and in some cells of the Moderate and High genotypes, almost all chromosomes exhibit these threads (as seen in Figure 2f).

At metaphase I the bivalents transgress to the equatorial plate with centromeres facing the poles. In a few cases, we observed multivalent-like configurations due to interchromosomal connections as mentioned above (Figure 2f). At anaphase I, the half bivalents disjoin normally, whereas the univalents segregate randomly resulting in unbalanced chromosome numbers at later stages. Moreover, anaphase bridges and multiple lagging chromosomes appeared (Figure 2g) in the Moderate and High genotypes. At anaphase II, we observed anaphase bridges and chromosome stickiness (interchromosomal connections) (Figures 2g, h). In cells at the tetrad stage, we found mostly balanced tetrads (9+9+9+9) in the Low genotype, while in the Moderate and High genotypes, tetrads with unbalanced chromosome numbers like (9+9, 9+8+1) and (10+8, 10+8) were observed (Figure 2i).

Number of chiasmata bonds in the three genotypes

Since the Moderate and High genotypes demonstrate relatively high numbers of non-chiasmatic chromosomes, we estimate the total number of chiasmata based on the numbers of counted rod and ring bivalents (with the assumption that they account for 1 and 2 chiasmata, respectively, Table 1, Figure 3b). Chiasma estimates for the Low, Moderate and High genotypes were 14, 13 and 14 per cell respectively, which is statistically not different ($P = 0.152$). Hence, we find no clear relation between the number of chiasmata bonds and the number of aneuploid offspring.

We therefore compared the mean number of estimated chiasmata in cells with univalent pairs with those that lack univalents. Our data suggests that the presence of univalent pairs leads to an increase of chiasmata on other chromosome pairs within the cell complement (Table 3).

More univalents in the Moderate and High genotypes

The frequencies of univalent pairs, rod bivalents, ring bivalents at diakinesis cells for the three genotypes are summarized in Table 1. The percentages of cells showing an expected number of nine bivalents are 89%, 61% and 57% for the Low, Moderate and High genotypes respectively. In most of the cells which contained univalents only one univalent pair was present, while in the Moderate and High genotypes, cell complements with two or more pairs of univalents were regularly observed. In the Low genotype, 7% of the cells have one pair of univalents and 4% of cells have two pairs of univalent. In the Moderate genotype, 27% of the cells have one pair of univalents and 12% of cells have two pairs of univalents. In the High genotype, 31% of the cells have one pair of univalents and 12% of cells have two or more pairs of univalents. To determine whether the three genotypes differ significantly in univalent incidence, a one-way ANOVA test on univalent counts showed significant differences between the genotypes ($P = 0.002$) (Figure 3a). In the Fisher's protected least significant difference test (series of pairwise t -tests) we showed

that the Low genotype differs significantly from both the Moderate and High genotypes, while the Moderate and the High genotypes do not differ from one another (Table 2).

Immunodetection of chiasma precursor MLH1

We further investigated the nature of the chiasmata bonds by performing an immunofluorescence detection with antibodies against the chiasma precursor MLH1 on spread diakinesis cells. MLH1 is known to occur in the later recombination nodules of the synaptonemal complex at pachytene and is involved in the class I crossover pathway. The MLH1 foci in our slides clearly correspond to most of the chiasma sites (Figure 4) but are lacking in some places that may represent sites of class II crossovers or class I crossover sites that were not detected by the immune fluorescent method. We analysed at least 15 cells for each genotype showing on average 10.93, 10.43 and 9.52 MLH1 sites per cell in the Low, Moderate and High genotype, respectively. Compared to the number of chiasmata bonds, we calculated the percentage of MLH1 stained chiasmata at 77.5 % for the Low and the Moderate type, and 69 % for the High genotype (Table 1).

Interchromosomal connections

Besides univalent formation, the presence of bivalent connections is another remarkable feature of this cauliflower. We counted numbers of connections between bivalents (Table 1). The average numbers of connections were 1.1, 2.0 and 2.1 in the Low, Moderate, and High genotypes, respectively. A one-way ANOVA suggests these differences in bivalent interconnections between the three lines are significant (P-value < 0.001), and a subsequent Fisher's protected least significant difference test indicates that the Low genotype differs significantly from both the Moderate and High genotype (P < 0.001). However, the difference between the Moderate and High genotype is not significant (0.5286). Comparison of the three lines for the correlation of numbers of crossovers and numbers of interchromosomal connectives (Figure 3c) revealed that both variables were not significant (*Kendall's Tau coefficient*: Low = 0.982, Moderate = 0.834, High = 0.375).

Repetitive DNA FISH analysis of diakinesis / metaphase I

To investigate if interchromosomal connections contain repetitive sequences, as was previously suggested by Pedrosa et al. (2001) in the study of *Ornithogalum longibracteatum* (*Hyacinthaceae*). Therefore, we performed a FISH experiment with repetitive DNA: 45s rDNA and two *Brassica* specific centromere repeats. The result indicated that 45s rDNA as well as the two *Brassica* centromere repeats painted interchromosomal connections (Figure 5a, c). Centromere and 45S rDNA specific sequences involved around 70% of bivalent connections, while less than 30% of the connections comprised non-stained chromatin. When 45s rDNA connections were observed, one or both chromosomes involved carry a 45s rDNA locus, whereas when centromere repeats are involved, these connect the centromere regions of two homologues (Figure 5b, d).

We then asked as to whether chromosomal connections are random, or whether specific chromosomes are more often involved in interconnections. To this end we had a closer look at the interconnections. Cauliflower has nine 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 for a subset of 19 painted interconnections (see Table 4). The theoretical chances of connections between chromosomes (while assuming no preferential connections being formed) are given by the following chances: $P(45s-45s) = 2/9 \times 1/8 = 1/36$, $P(45S-non) = 2/9 \times 7/8 + 7/9 \times 2/8 = 14/36$ and $P(non-non) = 7/9 \times 6/8 = 21/36$. A computed χ^2 test of 27.8, and $p_{df=2} < 0.001$, clearly shows a significant overrepresentation of 45S rDNA repeats in interchromosomal connections (Table 5). 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 the APETALA1/ CAULIFLOWER Arabidopsis mutant

Since cauliflower is of all Brassica crops most plagued by aneuploidy in its offspring, we wondered whether the genes responsible for curd formation indirectly cause aneuploidy among offspring. We therefore studied the meiosis of the *APETALA1/ CAULIFLOWER* double mutant of *Arabidopsis* that, like Cauliflower, displays the typical curd phenotype (Figure 6a). Microscopic observations of pollen mother cells at diakinesis in this double mutant showed interchromosomal connections (Figure 6b, c), albeit far less than in Brassica: in less than 10% of the analysed cells. Univalents were not observed neither did these plants produce aneuploid offspring. We conclude that *APETALA1* (AP1) and *CAULIFLOWER* (CAL) genes do not cause partial desynapsis during meiosis stages in *Arabidopsis*.

Discussion

Crossover recombination

In this study we aimed to explore whether aneuploidy in cauliflower (*Brassica oleracea* L. var. *botrytis*) progeny is caused by irregularities in chromosome pairing, recombination, and segregation during meiosis. The underlying assumption was that aneuploid gametes are the most likely source of aneuploids in the offspring. We therefore analysed male meiosis in spread pollen mother cell preparations of three cauliflower genotypes, Low, Moderate and High, that differ in percentages of aneuploid offspring. All genotypes display regular chromosome pairing at pachytene, while cells at diakinesis – metaphase I can contain one or few univalent pairs, a phenomenon known as partial desynapsis. We observed that in the Low and High genotypes, even 40% of cells contain such univalent pairs. Most of the cells show one univalent pair, but in the Moderate and High genotypes we often saw more univalent pairs. Univalents that move to either pole or remain lagging in the equatorial cell plane may lead to unbalanced chromosome numbers at tetrad stage of which only spores with 18, 19 or 20 chromosomes remain, of which the aneuploid cells will give rise to offspring plants with aberrant

aneuploid phenotype. Meiotic aberrations therefore seem causal to the occurrence of aneuploids in offspring.

The number of univalent pairs in the male meiocytes is clearly higher in the Moderate and High genotypes, but the difference between these numbers is not reflected by the differences in aberrant offspring between the Moderate and High type cauliflowers. We realise that sample size of the analysed meiotic cells is relatively small as slides with high quality cell spreads at different meiotic stages is time consuming and laborious. In addition, we have not been able to account for eventual effects of developmental, epigenetic, and environmental conditions that may influence the incidence of univalents. Although we have no cauliflower plants with only normal meiosis and balanced chromosome segregation and euploid gametes that can be used as a control, based on recent genetic studies, we can predict the number of chiasmata derived from estimated genetic map lengths for a *Brassica oleracea* with euploid offspring. According to Zhao et al. (2016) the total map length of cauliflower is estimated at 890 cM, which corresponds to an estimated crossover event number of $890/50 = 18$ per diploid cell complement, which equals on average two crossover events per chromosome. Yu et al. (2019) found comparable values for broccoli, which has an estimated map length of 799 cM, which equals 16 per cell complement and 1.78 c.o. per chromosome, respectively. These estimates are slightly higher than the partial desynaptic genotypes in this study. The slightly lower chiasma estimates here can partly be explained by incidental neighbour double chiasmata which have been interpreted as a single chiasma event. A second point of attention is that, for practical reasons, we focus on the course of male meiosis as cytology of female meiosis is almost impossible. We realize that most of the aneuploid gametes get lost in competition to euploid gametes in its way through pollen tuber to egg cell, a process known as certation (Heribert-Nilsson 1920; Koornneef and Van der Veen 1983; Janse 1987). This selection mechanism is known to reduce male aneuploid gamete transmission to values often far below 5%. It is therefore likely, that a significant rate of the aneuploids that we observed in the offspring families originated from aneuploid female gametes.

As basic processes in chromosome pairing, recombination and segregation are the same between male and female meiosis, apart from some recombination frequency differences between male and female meiosis (de Vincente and Tanksley 1991; Drauoud et al. 2007), we nonetheless consider partial desynapsis the primary cause of irregular chromosome segregation both in male and female meiosis of cauliflower. The question now arises as to whether univalent pairs always involve the same chromosomes. Chromosome painting with pooled BACs of *Arabidopsis thaliana* on mitotic chromosome spreads of this material support the observation that different chromosomes are involved (Ji, 2014).

We further elaborated on the number of crossover events in a series of MLH1 immunolabelling experiments on cell spreads of the Low, Moderate and High genotypes. MLH1, which is involved in the ZMM interference dependent pathway, produce class I crossovers, resides in the late recombination nodules of the synaptonemal complex at pachytene and remains a detectable protein in chiasmata until diakinesis / metaphase I. The immunofluorescent foci revealed that 77.5%, 77.5% and 69% of the chiasmata of the Low, Moderate or High genotypes are MLH1 positive (Table 1). The MLH1 negative

chiasmata are supposed class II crossover events or are supposed to be chiasmata in which the MLH1 could not be detected. The frequencies of these MLH1 foci correspond well with the estimated frequencies of 80-85% in *Arabidopsis* (Copenhaver et al., 2002; Chelysheva et al. 2010) and 70% in tomato (Lhuissier et al., 2007).

The relation between the number of univalent pairs and average number of chiasmata bonds and MLH1 foci per cell was surprising. While the Moderate and High genotypes display almost four times more univalents, the average number of chiasmata bonds remained largely the same (Table 1). If we compare the number of chiasma bonds in cells with and without univalents we see a much higher number of chiasma bonds in cells with univalents than in cells without (Table 3), suggesting that chromosome pair(s) that lack the obligate crossover compensate specific processes of crossovers placements *in trans*, thus keeping the total number of chiasmata in the cell the same, irrespective univalents or not. Apparently, reduction in crossing overs in one or more chromosomes is accompanied by a concomitant increase of crossovers in other chromosomes in the cell, a phenomenon described for maize partial asynaptic inbred lines of maize and reviewed by Sinha and Mohapatha (1969). The authors argue that a counteracting interchromosomal chiasma compensating mechanism is driven by chiasma interference, which is explained by assuming a definite quantity of material that is essential to chiasma formation in that cell. Carlton et al. (2006) proposed that meiotic progression is delayed on a per-nucleus basis until key events such as recombination have been completed. The apparent action of this checkpoint between early and late pachytene suggests that this transition is a target for cell cycle regulatory machinery. Termolino et al. (2019) described a comparable phenomenon for a paracentric inversion heterozygote in *Arabidopsis* where crossover suppression in the rearranged chromosome segment is associated with a significant increase of crossovers in the other chromosomes, thereby maintaining the total number of crossovers per cell unaffected. Such a trans regulation mechanism was found restricted to only male meiosis. In chromosome rearrangements of *Drosophila*, a similar interchromosomal compensation was observed causing a pachytene delay and hence influenced the pachytene checkpoint activity by extending to make more crossovers (Joyce et al. 2011).

Interchromosomal connections

The second striking feature of the partial desynaptic cauliflower genotypes is the presence of interchromosomal connections that were most noticeable in diakinesis and metaphase I cells. We found in each cell at least one of these chromatin threads, while in the Moderate and High genotypes on average two of such connections were observed. Such chromatin threads were different from the non-homologous centromere fusions seen in pachytene, a phenomenon that resembles the previously described non-homologous association in meiotic prophase I chromosomes of *Beta vulgaris* and are regular features of pachytene chromosomes (de Jong and Stam 1985). Moreover, we did not find MLH1 foci on these threads suggesting that such chromatin threads are not part of distal euchromatin segments, where chiasmata are located. In a series of chromosome painting experiments with FISH using probes of 45S rDNA and centromere repeats, we were able to demonstrate that the Brassica specific pericentromere repeats and the 45s rDNA repeat are involved, and that the NOR chromosomes containing

tandem arrays of this repeat display more connections than expected by chance. Furthermore, when centromere sequences were involved, they always connected the centromere regions of different chromosome pairs. An explanation for such connections is still not clear.

The chromosome connections, sometimes referred to as chromosome stickiness in the literature have been described in interspecific hybrids and inbred lines and may be the result of genetic mutations or environmental factors, which later leads to unequal distribution of genetic material to the daughter cells (Kláštorská and Natarajan 1975, Rayburn and Wetzel 2002). Other reports claim that such interconnections 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), abnormal chiasma formation (Higgins et al. 2005) and lead to dicentric anaphase bridges (Basi et al. 2006). Koul and Nagpal (2016) observed inter-telomeric connections in an achiasmatic meiosis in *Tradescantia spathacea*, in which the connections were prominent at early stages but disappeared at the time of diplotene. It was proposed that alteration in the cellular microenvironment may have altered the telomeric architecture that not only impeded the chiasmata formation but also induced ectopic pairing through inter-telomeric connections. Also, in our cauliflower material it is tempting to assume that interchromosomal connections are the results of ectopic pairing and/or erroneous DNA repair as meiotic double strand breaks in tandem repeat chromosome regions. That also the *APETALA1* – *CAULIFLOWER* double mutant of Arabidopsis demonstrates such interchromosomal connections, albeit at only 10% of the cells, suggests that the cauliflower curd phenotype leads to cell stress resulting in local ectopic pairing and DNA repair anomalies, but that the disturbed physiology does not lead to chromosome pairing and chiasma problems.

Features and inheritance of desynapsis

Desynaptic mutants belong to the most common synaptic disturbances in higher plants and have been described for a large number of species (reviews in Koduru and Rao, 1981; Kaul and Murthy, 1984; Wani and Bhat, 2017). Most of these mutants were found in natural populations, others were induced by ionizing radiation (Konvinčka and Gottschalk, 1971; Basi et al. 2006) or EMS treatment or were selected in a T-DNA (Perison et al. 1997) or transposon tagged populations (Cai and Makaroff 2001). In general, desynaptic mutants inherit as a single recessive mutant gene (Beadle 1930; Menzel and Brown 1955), sometimes by multiple genes (Rees, 1961) and were often selected from a group of plant that exhibit male sterility or vary largely in the shape and size of euploid, aneuploid and unreduced pollen grains. The majority of the desynaptic mutants affect the entire chromosomal complement but a few influences only specific chromosomes. A striking phenomenon found in desynaptic plants is the occurrence of chromosome stickiness or interchromosomal connections (Rao et al. 1990; Pagliarini et al. 2000; Mehra and Rai 1972; Kláštorská et al. 1976; Higgins et al. 2005), although their appearance differ between the species where they were observed. Rao (1975) found *Coix lacryma-jobi* demonstrated sterility by desynapsis only during the hot summer months and was supposed the result of genotype-environment interactions. The latter suggest that physiological and epigenetic mechanisms are involved in the control of meiotic processes leading to desynapsis.

How curd phenotype may induce partial desynapsis

Cauliflower which is known to have a longer inflorescence meristem stage during vegetative-to-reproductive transition than other *B. oleracea* varieties: the flower primordia will not mature but regenerate more inflorescence, which may induce physical stress on cauliflower. The most related broccoli (*B. oleracea* L. var. *italica*) and 'Romanesco' broccoli have comparable curd phenotype but differ with cauliflower in the rate of branch primordia production and the iteration intervals (Kieffer et al. 1998). As curds of broccoli are arrested young flower buds, curds of cauliflower and Romanesco is arrested inflorescence (Azpeitia et al 2021). These dissimilarities point at fundamental developmental differences at why cauliflower can produce aneuploid offspring, whereas broccoli and Romanesco hardly do not. Research in *Arabidopsis* has hinted towards possible differences between the species.

Previous studies in *Arabidopsis* indicated that the *FRUITFULL*, *APETALA1* and *CAULIFLOWER* genes play a pivotal role in the inflorescence architecture (Ferrándiz et al. 2000), and that the *APETALA1* and *CAULIFLOWER* double mutant produces inflorescence meristems with the cauliflower phenotype. The close evolutionary relation of *B. oleracea* with *Arabidopsis thaliana* (Liu et al. 2014), was our reason to test chromosome aberrations in the *APETALA1* and *CAULIFLOWER* double mutant with the curd like phenotype (Bowman et al. 1993). Azpeitia et al. (2021) elaborated the cauliflower curd phenotype in *Arabidopsis* by modelling and experimentally validated the gene regulatory network involves the *SAX*, 'API' for *API1/CAL*, *LFY* and *TFL1* genes to deduce the fractal nature of cauliflower curd spiral patterns. The arresting period in inflorescence development might cause delay or interruption of (pre-)meiotic processes, which leads to partial desynapsis. The authors speculated that similar genetic processes could underlie both morphologies. According to Duclos (2008), is the arrest of cauliflower flower developmental stage regulated by the *FRUITFULL (FUL)*, *TERMINAL FLOWER 1 (TFL1)*, *CAULIFLOWER CURD EXPRESSION 1* AND *REPRODUCTIVE MERISTEM 1* genes that are recognized as meristem identity genes pointing at their involvement in cauliflower curd arrest (Shannon and Meeks-Wagner, 1993; Ferrandiz et al., 2000, Lee et al. 2000). The action of *FUL* might be related with controlling development stage of curd formation while *TFL1* is recognized as a factor of curd enlargement (Duclos et al. 2008). Both are identified to be special associated with cauliflower curd formation by Palmer (2001) and Franco-Zorrilla (1999). These genes may involved meiosis regulation and so, seem more likely play a role in the partial desynapsis during maintaining curd arresting.

What we can conclude from this study is that partial desynapsis induced abnormal meiosis which cause aneuploid gametes and so to aneuploid cauliflowers. The typical curd causes a physiological stress in the development of the young floral meristems and flowers may induce epigenetic changes which causes cues in the time scale of early flower developmental processes (Yaish et al., 2011), including those that are involved in synapsis and maturation of the chiasmata. Such an epigenetic involvement was also suggested in the study of Chable et al. (2009) who demonstrated decreased global DNA methylation rates in cauliflower plants that exhibited an evolution of their phenotype during vegetative cycle. The interchromosomal connections that we and others found in the desynaptic cells point at erroneous DNA repair in tandem and other repetitive sequences in the interphase nuclei keeping

the neighbor chromosomes connected by thin chromatin threads. The implications of the desynapsis in cauliflower may be frustrating for breeders as selecting for bigger or fast growing curds intrinsically means a higher physiological stress in the curd tissue and early flower development, and hence a higher change for aneuploid rate in the gametes.

Abbreviations

AP1:	APETALA1
BSA:	Bovine Serum Albumin
<i>CAL</i> :	the gene <i>CAULIFLOWER</i>
Cy5:	Cyanine 5 fluorochrome
DAPI:	4',6-diamidino-2-phenylindole
DEAC:	7-Diethylaminocoumarin-3-carboxylic acid
FISH:	Fluorescence <i>in situ</i> hybridization
FITC:	Fluorescein isothiocyanate
<i>FUL</i> :	the gene <i>FRUITFULL</i>
<i>MLH1</i> :	mutL homolog1
PBS-T:	Phosphate buffered saline with 0.1% v/v Triton
PMCs:	pollen mother cells
SSC:	Standard Saline Citrate
TFL1:	TERMINAL FLOWER1
TNB:	Tris-HCl pH7.5, 0.15M NaCl, 0.5% blocking reagent

Declaration

Author contribution. All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written by Xianwen Ji and Hans de Jong and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Overview of rod-, ring- and univalent numbers per cell, including chiasma estimates, MLH1 foci and interchromosomal connections for the Low, Moderate and High genotypes.

	Low	Moderate	High
number of cells	57	59	52
total number of chromosomes	1026	1062	936
total number of ring II	303	295	288
average number of ring II per cell	5.32	5.00	5.54
total number of rods	201	203	152
average number of rods per cell	3.53	3.44	2.92
total number of univalent pairs	8	32	28
average number of univ. pairs per cell	0.14	0.54	0.54
total number of chiasmata bonds	807	691	728
average number of chiasmata bonds per cell	14.10	13.46	13.80
number of cells for immunofluorescence	15	61	33
total number of MLH1	164	636	314
average number of MLH1 per cell	10.93	10.43	9.52
Percentage MLH1 / chiasmata bonds	77.5	77.5	69.0
number of cells with interchromosomal connections	51	59	51
number of interconnectives	51	115	107
average number of interconnectives per cell	1	1.95	2.1

Table 2. Fisher's protected least significant difference test on univalent prevalence among all cells observed in the three genotypes

Comparison (univalent)	Difference	Lower 95%	Upper 95%	t	Probability	Significant
Low vs Moderate	-0.3981	-0.7028	-0.0935	-3.091	0.0023	Yes
Low vs. High	-0.402	-0.6971	-0.107	-3.223	0.0015	Yes
Moderate vs. High	-0.0039	-0.3061	0.2983	-0.031	0.9756	No

The low genotype is significantly different from the Moderate and High genotypes: $p: 0.0023 < 0.05$ and probability: $0.0015 < 0.05$, while the Moderate and High genotypes do not differ significantly in univalent frequency (probability: $0.9756 > 0.05$).

Table 3. Average numbers of ring and rod bivalents, and number of chiasmata in cells with and without univalents.

	With univalents			Without univalents		
	Low	Moderate	High	Low	Moderate	High
Number of cells	51	36	30	6	23	22
Ring bivalents	5.5	5.4	5.8	3.2	4.3	5.2
Rod bivalents	3.6	3.6	3.2	4.5	3.2	2.5
Chiasmata bonds (estimated)	14.6	14.4	14.8	10.9	11.8	12.9

Table 4. Fisher's protected least significant difference test on bivalent interconnections among three genotypes

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

The Low genotype is significantly different from the Moderate and High genotypes: probabilities are less than 0.05, while the moderate and bad lines do not differ significantly in bivalent interconnections (probability: $0.5286 > 0.05$).

Table 5. The localization of 19 bivalent interconnections that comprised 45s rDNA. Please see text for explanation.

Interchromosomal connection	Observed	Expected
45S-45S	4	0.513
45S-non	10	7.41
Non-non	5	11.02

Figures

Figure 1

Photomicrographs of DAPI stained pollen mother cells of cauliflower, *B. oleracea* L. var. botrytis, $2n=2x=18$, at different meiotic stages. Bar equals 10 µm in the figures. (a) Cell complement at pachytene of the High type. The homologous chromosomes are clearly almost fully paired. The cells show a typical pachytene morphology with brightly fluorescing pericentromere regions, which cluster at some places (arrows). The weaker fluorescing euchromatin has the typical pattern of smaller chromomeres. (b) Cell of the High type at late pachytene/ early diplotene, where only Nucleolar Organizer Region and few other chromosome regions have disjoined (arrow heads).

Figure 2

DAPI stained pollen mother cells at diakinesis – anaphase II. The figures (a) and (b) are from “Low genotype material; the figures (c), (d) and (i) are from the Moderate genotype; the figures (e), (f), (g), (h) are from the High phenotype. (a) Cell at diakinesis – metaphase I with nine bivalents. Ring-bivalents have at least one chiasma on both chromosome arms. Open rings have distally located chiasmata on both arms; compact rings have chiasmata closer to the centromeres. Rod-bivalents have (mostly one) chiasma on one of the chromosome arms (example of a rod bivalent is shown). (b, c) Cell with clear examples of interchromosomal connections (arrows). “p” refers to chiasma that is more proximal (closer to the centromere), and “d” more distal (d) Cell with two univalents, of which apparently either is associated with a nearby bivalent. (e) In this cell two bivalents are tightly connected, giving the impression of a quadrivalent. (f) Metaphase I cell with two univalent pairs (I-pairs) shown with thin arrows. (g) Anaphase I cell with 10 visible univalents of which one may be a univalent chromatid. (h) Anaphase II showing two lagging chromosomes with interconnection. (i) Anaphase II cell with one lagging chromosome. Two of the anaphase II complements are on top of each other. The segregation of 8+9+1+18 chromatids is clearly unbalanced.

Figure 3

(a) Pie charts presenting the proportions of ring bivalents, rod bivalents and univalent pairs in the Low, Moderate and High genotypes of cauliflower, *B. oleracea* L. var. botrytis, $2n=2x=18$. (b) Box and whisker plots of the number of chiasmata bonds of the Low, Moderate and High genotypes. (c) Scatter plot showing number of interchromosomal connections and number of chiasmata bonds for the Low,

Moderate and High genotypes. The trendlines for each of the genotypes demonstrates that interchromosomal connections and number of chiasmata are statistically not significant.

Figure 4

FISH probed with labelled repetitive sequences on spread pollen mother cells at diakinesis of the High genotype. (a) and (c) 2-colour FISH with the DAPI imaging and the blue 45S rDNA foci; (b) and (d) 4-colour FISH with the red fluorescence signals of the Brassica specific centromere repeat Centbr1 (pseudo-coloured in red) and green fluorescence of the Brassica specific centromere repeat Centbr2. The arrows point at connections between chromosomes that colocalize with the 45S rDNA and centromere repeats.

Figure 5

Immunofluorescence with the MLH1 AB on diakinesis cells of (a) Low type, (b) Moderate and (c) High genotype. MLH1 foci are supposed to demonstrate sites of late recombination nodules, the place of crossover events. The number of MLH1 foci is lower than expected possibly due to inaccessible target sites. For better contrast DAPI images have been displayed in red.

Figure 6

(a) The AP1/Cau double mutant of *Arabidopsis* forms a typical curd phenotype resembling that of cauliflower. (b) and (c) show two photomicrographs of meiotic pollen mother cell of the AP1/Cau mutant at diakinesis. Interconnections between bivalents are clearly observed (arrows) whereas univalent pairs are completely lacking.